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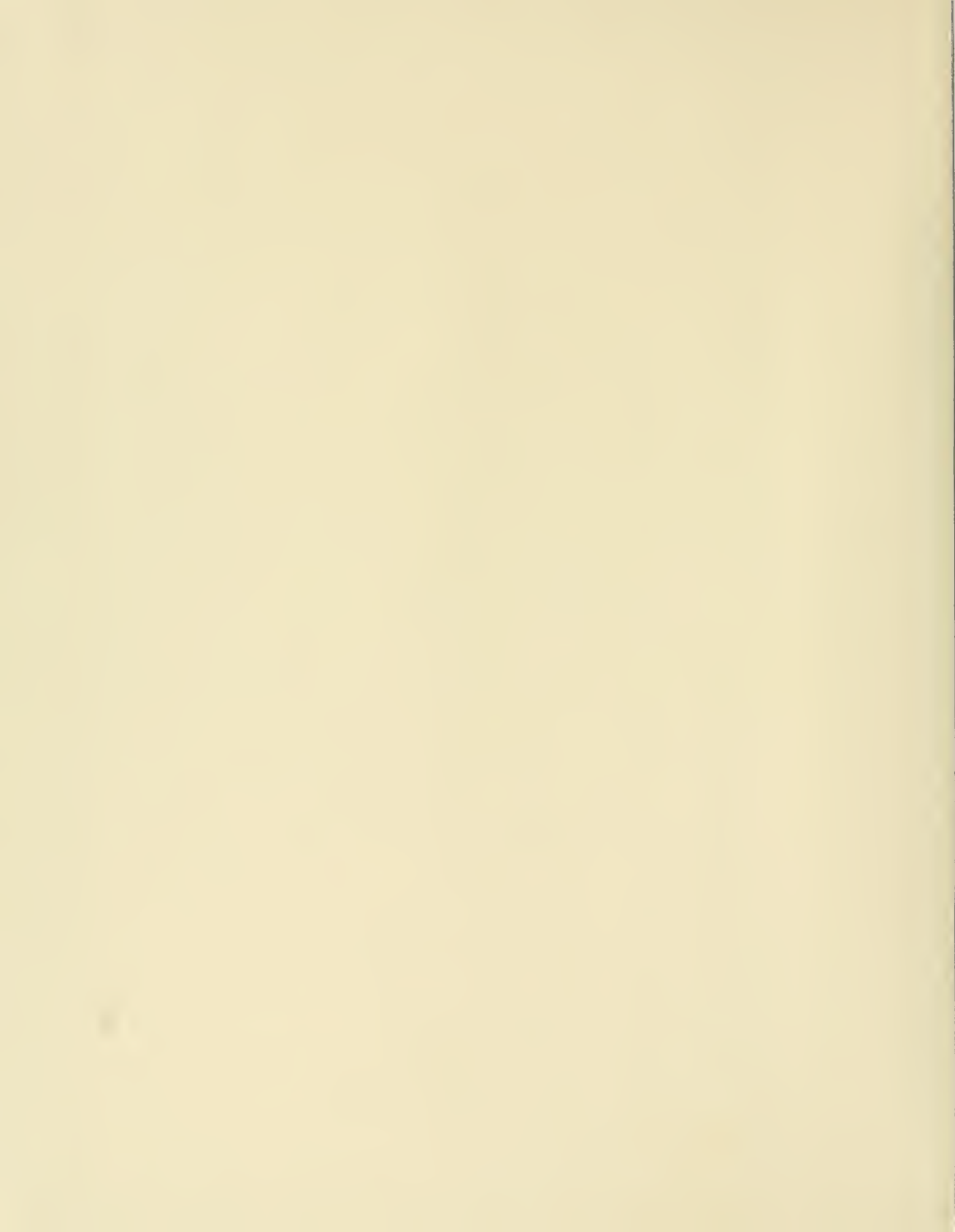


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CHROMATOGRAPHIC ANALYSIS OF DITHIOPHOSPHATE MODIFIED DNA

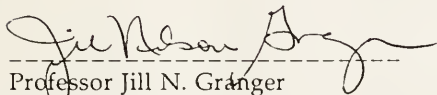
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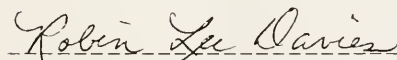
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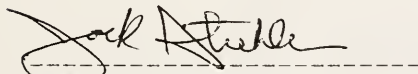
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**CHROMATOGRAPHIC
ANALYSIS OF
DITHIOPHOSPHATE
MODIFIED DNA**

*Senior Honors Research Thesis
Department of Chemistry
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INTRODUCTION

Today, synthetic oligonucleotides (oligos) have many well known biochemical applications, including use in recombinant DNA techniques, use as hybridization probes, and capabilities as antisense and anti-viral drugs¹. In the 1970's, synthetic oligos were first used by Zamecnik and Stephenson in an anti-viral study on the Rous sarcoma virus². Since then, synthetic oligos have been studied in the hopes of developing therapeutic drugs to cure cancer and HIV as well as many other diseases.

In normal adult cells, regulation of cell growth is maintained by a more or less steady state of tissue growth with cells dividing as frequently as dying. However, a cell may start growing out of control and become tumorigenic. This is caused by an oncogene, a mutant form of a normal gene involved in growth control. Transformation of the gene from a proto-oncogene to an oncogene can occur through many methods including promoter insertion or mutation. Most cancer cells are formed because of the effect of one of five types of oncogene proteins on its normal cell predecessor: growth factors, growth factor receptors, intracellular signal transducers, nuclear transcription factors, and cell-cycle control proteins. These oncogenic products all work in different ways and can effect changes in cell growth so as to transform the gene from a proto-oncogene to an oncogene. The majority of the mRNA in the transformed cell is the same as in a normal cell. The mRNA which is different is responsible for synthesizing the proteins which prove harmful to the body³.

¹ Ho, Peter T C & David R. Parkinson. *Seminars in Oncology* **24**, 2 (1997) p.187-202

² Zamecnik, PC & ML Stephenson. *Proc Natl Acad Sci USA* **75**, (1978) p 280-284

³ Lodish, Harvey; David Baltimore, Arnold Berk, S. Lawrence Zipursky, Paul Matsudaira & James Darnell. Molecular Cell Biology 3rd Ed. New York. Scientific American Books, Inc; 1995

Many traditional drugs, including intercalators, which interact with the DNA of the transformed cell have been synthesized. However, these drugs cannot target specific gene expression because they show no sequence binding specificity and often create side effects by inactivating other proteins or DNA molecules^{4,5}. Many thousands of proteins can be synthesized by a single mRNA, so traditional drugs must be taken in an extremely high dose which can cause cell toxicity.

Antisense oligos may prove more successful in the treatment of cancer by attacking the mRNA of a cell and therefore should require a lower dose while exhibiting greater specificity. The synthetic antisense oligo is comprised of nucleic acids complementary to the mRNA sense strand. In principle, the oligo enters the cell and binds tightly to the messenger RNA or the DNA and inhibits translation or transcription. There are many theories as to the mechanism used by antisense oligos to block the cell's ability to express the targeted gene as shown in figure 1⁶.

⁴ Miller, Paul S. & Paul O P. Ts'o *Ann Rep in Med Chem* **23**, (1988) p 295-303

⁵ Rawls, Rebecca L. *C&EN* (June 2, 1997) p 35-39

⁶ Lönnberg, Harri & Eero Vuorio *Ann Med* **28** (1996) p 511-522

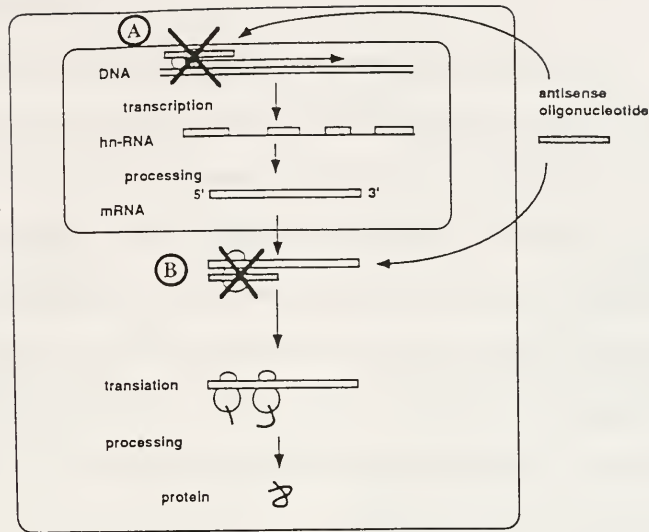


Figure 1: Possible mechanisms of antisense strategy⁶

The inhibition of transcription is the most recently studied mechanism and is called antigene therapy. Transcription is the synthesis of RNA, using DNA as a template. The antisense oligo hybridizes with a DNA:DNA duplex and forms a triplex. This inhibits the RNA polymerase from binding and the DNA from serving as a template for transcription⁶.

Antisense therapy, the inhibition of translation, has been the most widely studied and is the most common mechanism⁶. There are two different mechanisms by which the inhibition of translation could occur in the cell. The first mechanism involves the inhibition of the binding of the ribosomal subunits needed for translation (shown in

figure 2) and the second mechanism involves RNase H activation and the subsequent hydrolysis of the mRNA^{7,8,9}.

Initiation of translation is aided by initiation factors and begins with the association and binding of a Met-tRNA and small (40S) ribosomal subunit to the mRNA. The large (60S) ribosomal subunit then binds to this complex so that the Met-tRNA is located in the peptidyl (P) site. Elongation of the protein starts with an amino-acyl tRNA binding to its complementary mRNA codon at the amino-acyl (A) site in the 60S subunit. Transpeptidation occurs and the growing amino acid chain is covalently attached to the amino-acyl tRNA in the A site. The whole complex is then translocated down the mRNA sequence so that the nascent polypeptide chain is in the P site. This leaves the A site free to bind the next amino-acyl tRNA and the cycle continues until release factors trigger the release of the polypeptide chain¹⁰.

⁷ Crooke, Stanley T. *Annu Rev Pharmacol & Toxicol* **32**, (1992) p 329-376

⁸ Uhlmann, Eugen & Anuschirwan Peyman *Chem Rev* **90**, 4 (1990) p544-584

⁹ Imai, Enyu; Yoshitaka Akagi & Yoshitaka Isaka *Nephrol Dial Transplant* **12**, (1997) p 2213-2215

¹⁰ Voet & Voet. *Biochemistry*. 2nd Ed. Wiley; 1996 p 898

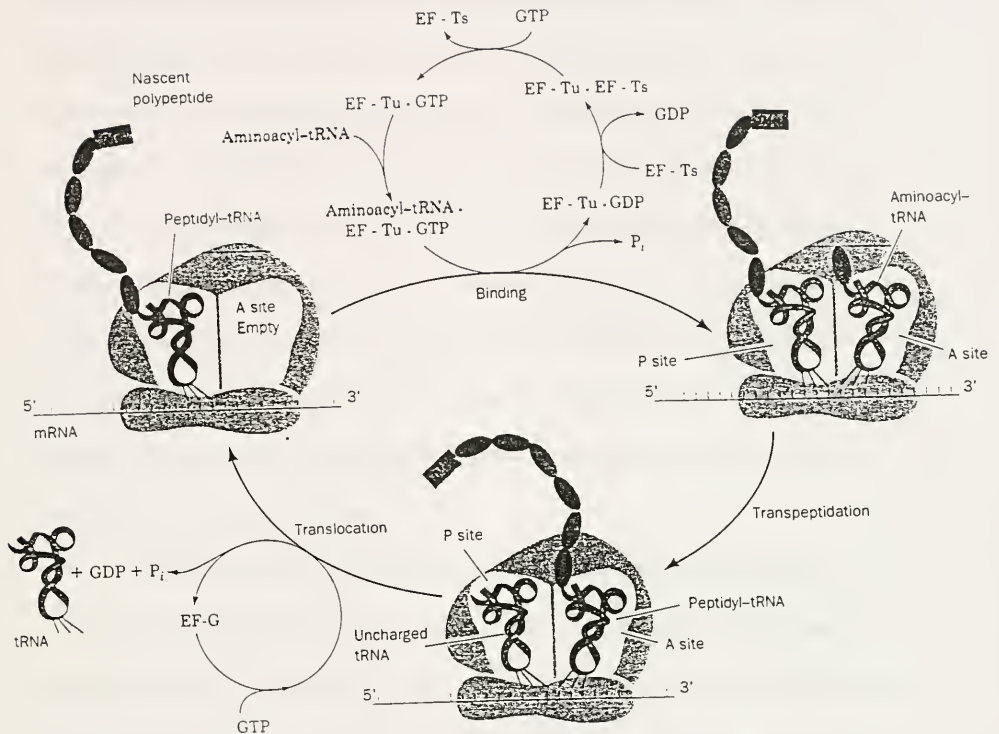


Figure 2: Mechanism of translation showing initiation, elongation, and release¹⁰

The first mechanism proposed for the inhibition of translation involves the inhibition of the initiation complex. By hybridizing to the mRNA at the ribosomal binding site, the oligo sterically blocks the ribosomal subunits from binding¹. Therefore the mRNA cannot synthesize the protein vital to the disease. For this mechanism, higher doses would be required as a new oligo is needed to block another mRNA from synthesizing proteins.

The second proposed mechanism of the inhibition of translation involves the recognition of the antisense oligo by an enzyme. As in the first mechanism, the antisense oligo hydrogen bonds by Watson-Crick base pairing to the complementary bases of the mRNA, thereby forming an RNA:DNA duplex. This duplex is recognized by RNase H, an enzyme that participates in DNA replication. RNase H degrades RNA attached to DNA¹¹. Since the RNA is hydrolyzed, the oligo is free to hybridize with, or attach to another mRNA. Since each mRNA can synthesize thousands of proteins, this means that each oligo has a catalytic effect and therefore can have a greater effect than more traditional treatments on stopping the synthesis of proteins^{6,8}. This also means that a smaller dose can be used as compared to traditional treatments and there is a better chance of controlling the disease.

The first oligos used in antisense therapy were unmodified phosphodiester linkages, and although they were successful at hybridizing to the mRNA, they were degraded too easily by nucleases within the cells. To combat this problem, modifications of the oligonucleotide structure were studied to provide the oligo with nuclease stability once inside the cell. In addition, modifications were chosen to assure chemical stability, to promote uptake into the cells, to promote selective hybridization to the specific mRNA, and to decrease side effects and cell toxicity as well as being cost effective⁶. The different types of modifications fall into three major categories: base, sugar and backbone modifications.

¹¹ Oberbauer, Ranier *The Middle European Journal of Medicine* 109, 2 (1997) p 40-46

Base modifications are different chemical alterations (i.e. methyl, bromo, or propynyl) on a base (A,C,G,T) in the synthetic oligonucleotide. Some examples of base modifications are shown in figure 3.

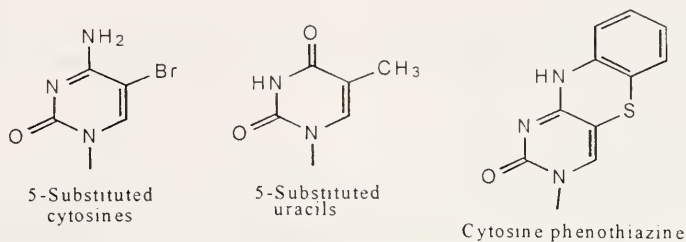


Figure 3: Examples of different base modifications of DNA⁶

These modifications have been shown to selectively hybridize to the target mRNA, but they require additional modification for stability against nuclease degradation⁶. The base modification also reduces or eliminates RNase H activity, thereby greatly decreasing the antisense effect on the cancer cell⁴.

Sugar modifications are modifications of the deoxyribose, typically at either the 5' carbon or at the oxygen in the ring (see figure 4).

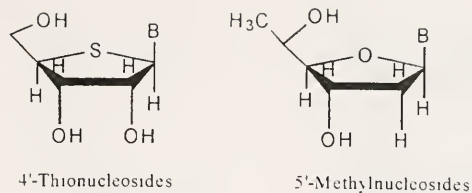


Figure 4: Examples of different sugar modifications of DNA⁶ B=Base

These modifications have been studied more and prove to have greater stability than base modified oligos and unmodified oligos against nucleases¹². However, sugar modifications also reduce the activity of RNase H meaning higher doses would need to be used to have an effect⁴.

Backbone modifications are the most well studied of these three general types of modifications. The backbone of DNA is composed of phosphodiester connecting each deoxyribose ring. Three of the most common backbone modifications are methyl, amine, and sulfur and can be seen in figure 5.

¹² Imbach, Jean-Louis, Bernard Rayner & François Morvan. *Nucleosides & Nucleotides* 8, 5&6 (1989) p 627-648

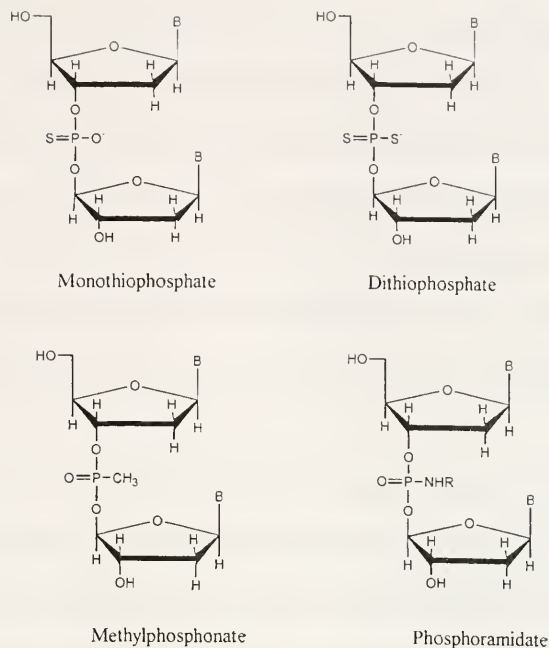


Figure 5: Common backbone modifications of DNA⁶

These modifications increase stability of the oligo against nucleases while the hybridization and selectivity of the mRNA are maintained. The sulfur modified oligos have shown the highest affinity of the lot¹³. Among these four types of backbone modifications, sulfur modified (or monothiophosphate modified) have been the most widely studied since it has shown increased affinity for the target mRNA as well as serving as the best substrate for RNase H⁴. Although the monothiophosphate modified (POS) oligos have increased stability against nucleases in the cell as compared to the unmodified DNA, they are still partially degraded. Monothiophosphate oligos also have

¹³ Ghosh, Mridul K, Krishnakali Ghosh, Otto Dahl & Jack S. Cohen. *Nucleic Acids Res* 21, 24 (1993) p 5761-5766

an optical, chiral center and will form diastereomers which can decrease the antisense effect of the oligo¹⁴. Therefore, dithiophosphate modified oligos (PS2) have been synthesized because they are modified achiral mimics of normal DNA. The studies of phosphorodithioates have shown that the sulfur modifications are chemically stable, are resistant to nuclease degradation, maintains stable and selective hybridization to mRNA, and serves as a good substrate for the RNase H degradation of mRNA⁴. The PS2 modification has also been shown to actively inhibit HIV-1 reverse transcriptase and viral replication¹⁵.

In order to approximate the anti-viral or anti-oncogenic effect of a specific oligo sequence upon cancer cells of a human, transformed cell cultures can be used. For anti-viral studies, assays are developed which monitor the reduced efficiency of virus production. Assays may also monitor the output of the protein being directly affected by the antisense oligo. For example, a tyrosine kinase assay is used to monitor the anti-viral effect of anti-RSV oligos on Rous Sarcoma virus (RSV) in chicken fibroblast cells. The RSV contains a gene, called *src*, which produces tyrosine kinase. If the oligo is binding and working correctly, this should be shown by a drop in the production of tyrosine kinase in the cells. An anti-oncogenic effect can be monitored by measuring cell toxicity over time. For example, the anti-*myc* oligo is designed to attach to mRNA in a human leukemia transformed cell line, HL60. As the mRNA is hydrolyzed, the protein that is produced by the gene decreases, eventually leading to the cell's death or cessation of

¹⁴ Cohen, Aharon S , Maria Vilenchik, Judith L. Dudley, Mark W. Gemborys & André J. Bourque. *J. Of Chrom.* **638**, (1993) p 293-301

¹⁵ Marshall, W S & M H. Caruthers. *Science* **259**, (1993) p 1564-1570

growth. By counting the number of living cells over time, the effect of the antisense oligo can be determined.

For these cell studies, specific sequences are needed to selectively hybridize to the target mRNA sequences. These sequences can be synthesized using an automated DNA synthesizer. To synthesize the normal phosphodiester oligo, each base is added in a cycle of four steps: detritylation, coupling, capping, and oxidation. A support that contains the base at the 3' end of the oligo to be synthesized is loaded onto the DNA synthesizer. The bases have all been protected with dimethoxytrityl (DMTr) and β -cyanoethyl groups to prevent unwanted reactions. The detritylation step uses acid to remove the hydrophobic dimethoxytrityl protecting group from the hydroxyl group on the 5' end of the sugar leaving it free to react in the coupling reaction. During coupling, the next deoxynucleoside is covalently attached to the hydroxyl group of the previous nucleoside by a condensation reaction. Tetrazole is present to catalyze the reaction. Acetylation caps the unreacted bases to prevent the extension of incorrect sequences. Oxidation, using iodine, of the phosphorous backbone from the phosphite triester to the phosphotriester completes the cycle and the process can begin again with the next base in the sequence¹⁰ (see figure 6).

The synthesis of the POS oligo requires a sulfurizing step in place of the oxidation step (see figure 7). In the literature and in this research, most of the POS oligos used for cell studies have a sulfur modification on every base in the sequence. Therefore, after each coupling, a sulfurizing reagent, such as Beaucage, is used to attach a sulfur to the phosphorous backbone. Any unreacted bases are then capped to eliminate sequence errors.

The synthesis of the PS2 oligo requires a sulfurization with POS as well as a monothiophosphoramidite. The monothiophosphoramidite is a nucleoside that already contains one thio modification on the phosphorous. The PS2 oligo can be modified between each base but is usually only modified at specific linkages between bases in literature and in this research, so the oxidation step will be used to form a normal phosphate backbone between the other bases. When a dithio modification is put in, the DNA synthesizer uses the monothiophosphoramidite in place of the unmodified base. Coupling occurs as in the synthesis of an unmodified oligo, and the sulfurizing reagent is used in place of the oxidation step (see figure 8). The unreacted oligos are then capped as described previously.

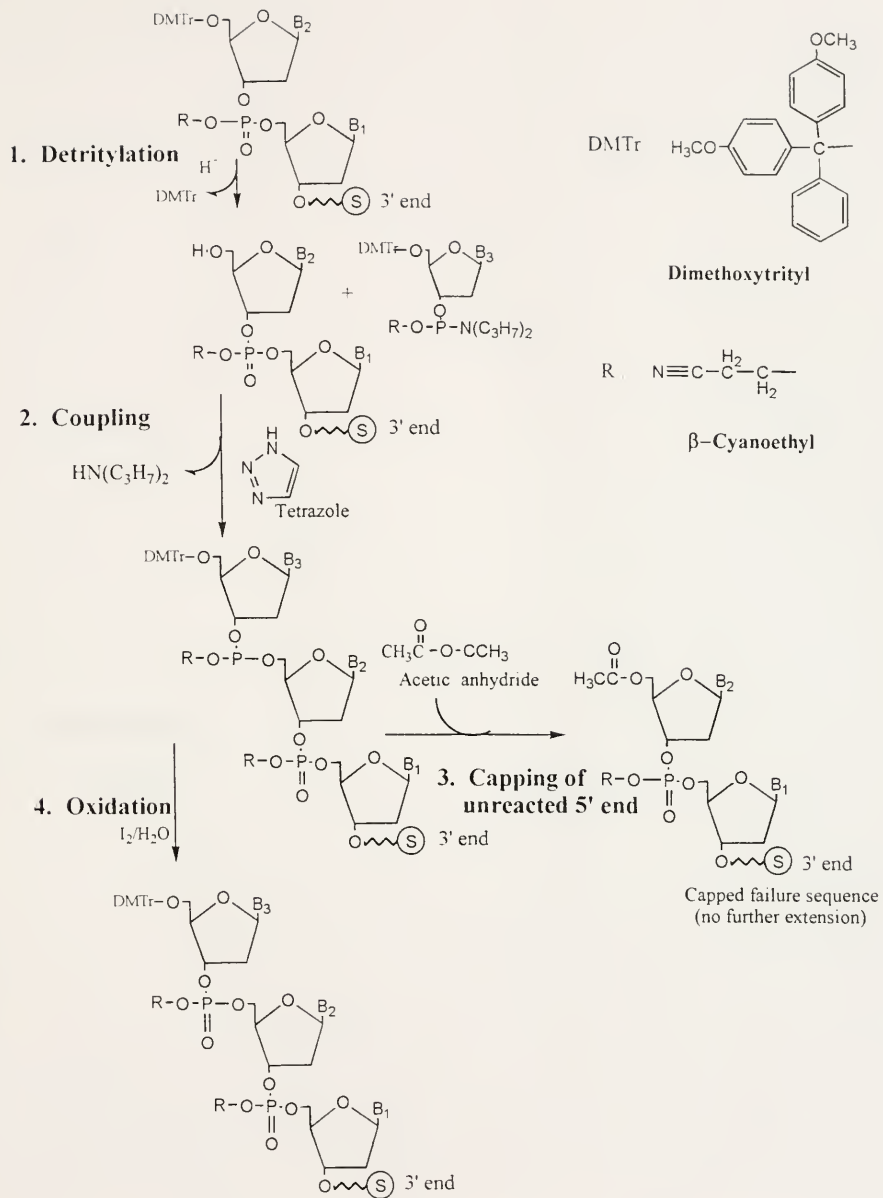


Figure 6: DNA synthesis scheme for unmodified oligos

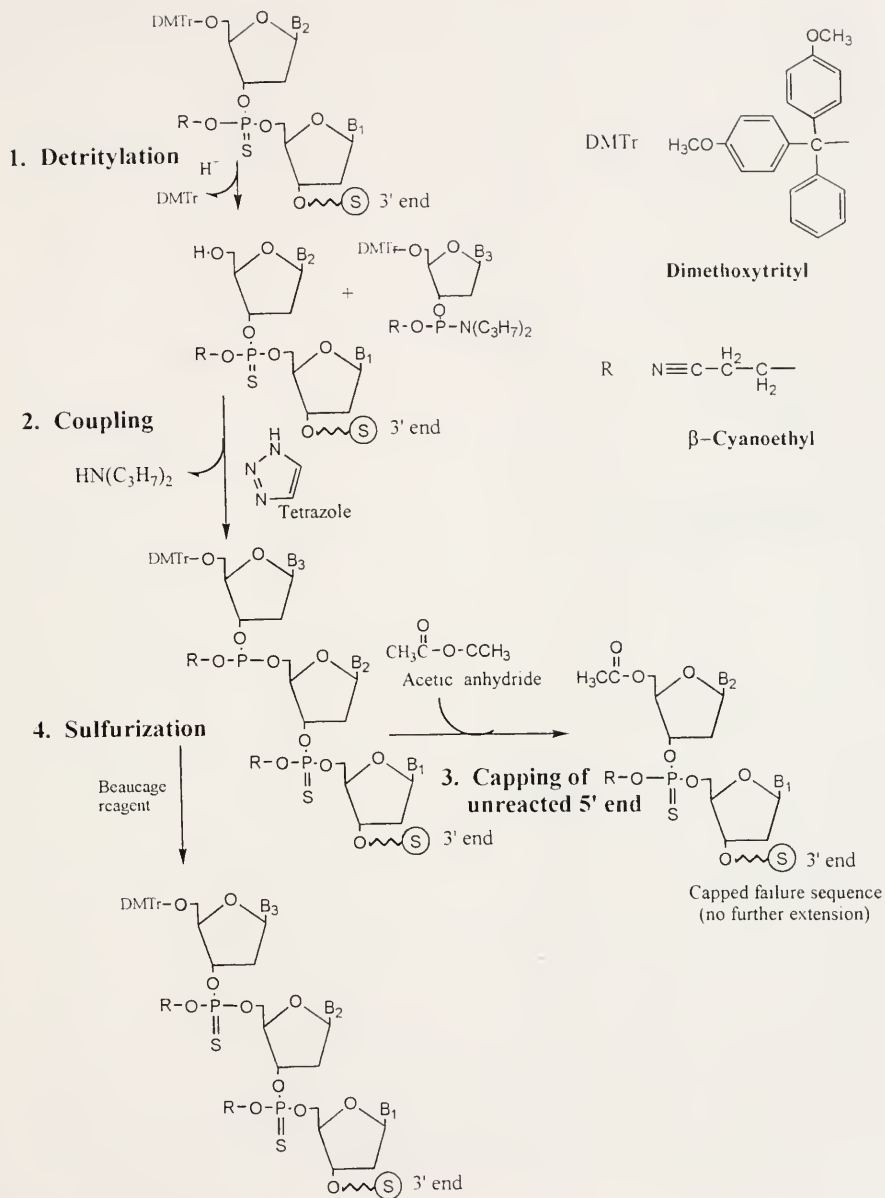


Figure 7: DNA synthesis scheme for a monothiophosphate (POS) oligo modified at every linkage

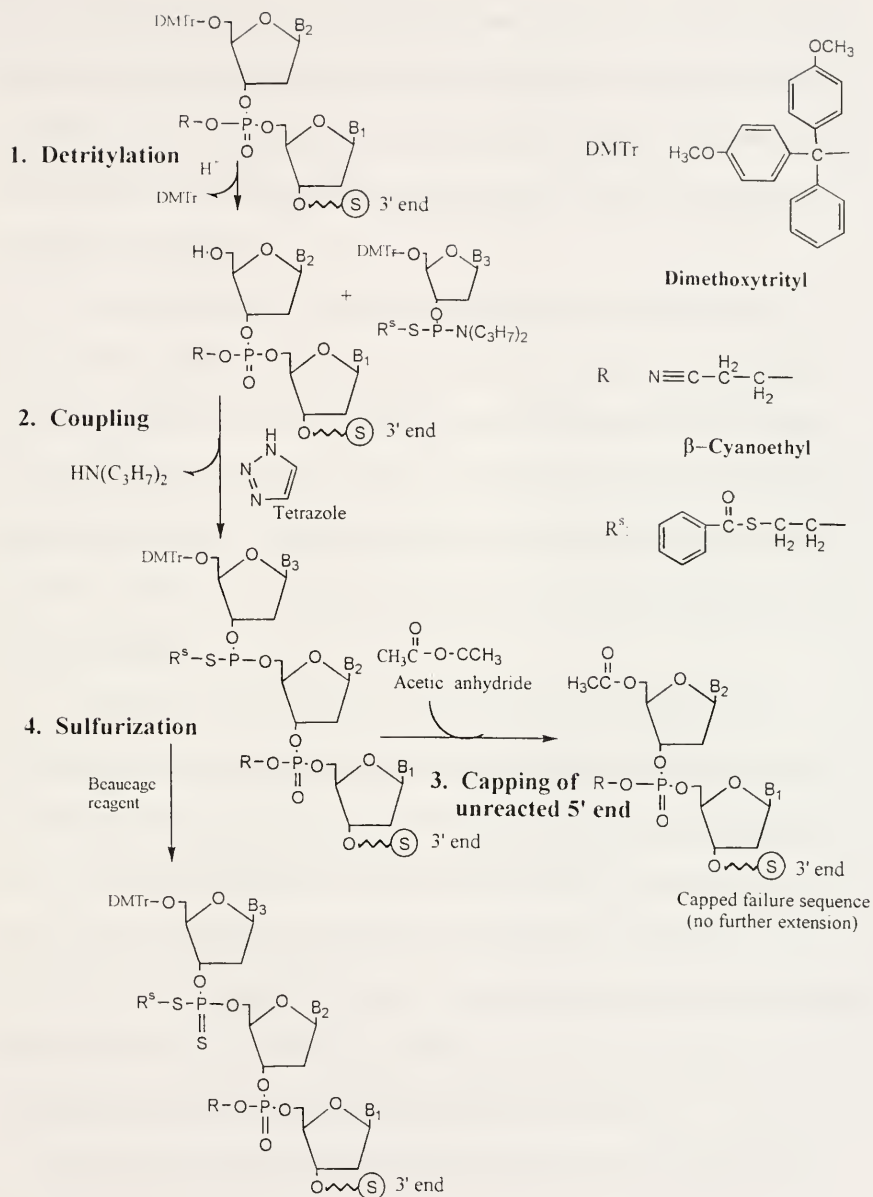


Figure 8: DNA synthesis scheme of dithiophosphate (PS2) oligo modified between B² and B⁴

To ensure that only the desired oligo sequence is present in the cell studies and not other contaminants or failure sequences from the synthesis, purification of the oligo is needed. There are three major methods of choice for purification of DNA oligos: gel electrophoresis, anion exchange chromatography, and reverse phase high performance liquid chromatography¹⁶.

In gel electrophoresis, an agarose or polyacrylamide gel in a buffer solution through which an electric current runs separates the DNA by size. The agarose gel provides a large, polysaccharide cross-linked matrix through which the DNA travels. The polyacrylamide gel provides a smaller, cross-linking matrix through which smaller oligos can be separated. When loaded into the wells of a gel in an electrophoresis box, the DNA will travel from the negative electrode towards the positive electrode when the current is turned on.

This is because DNA is polyanionic and is attracted to a positive charge. Throughout the electrophoretic process, separation of the DNA molecules occurs based on its molecular weight or size since all DNA molecules have a similar charge to mass ratio¹⁷. The larger pieces of DNA cannot navigate through the matrix as quickly as the smaller pieces of DNA so the smaller pieces travel down the gel faster. By running a standard ladder for which all DNA sizes are known, estimates of the sizes of the bands of DNA can be determined. The distance of migration of the bands is related logarithmically to the molecular weight of the DNA by this equation:

$$\text{Distance} = a - b \log_{10} \text{MW} \quad (\text{where } a \text{ and } b \text{ are empirically determined constants})$$

¹⁶ Warren, W. & M. Merion. *Biochromatography* 3, 3 (1988) p 118-126

¹⁷ Walker, J.M & E.B. Gingold. Ed. Molecular Biology and Biotechnology. 2nd Ed. Royal Society of Chemistry; 1988 p.27-30

However, there are a few disadvantages in using gel electrophoresis to purify the synthetic oligos. Gel electrophoresis results in low recovery of oligos. It is load limited so only a very few micrograms of oligos can be purified during each run. Electrophoresis is also a tedious process; the gel has to be cut up before the DNA can be extracted, which can cause contamination, and the oligos must be desalted, usually by dialysis, before use in cell studies^{14,18}.

Both anion exchange and reverse phase chromatography are forms of liquid chromatography. Liquid chromatography separates molecules as they flow across the surface of a column, called the stationary phase. The solution in which the molecules are solvated is called the mobile phase. As the molecules in the mobile phase flow through the column, some of the molecules will interact with the stationary phase and will partition. This partitioning results in each molecule eluting from the column at different times. The mobile phase must be changed in some fashion to create an environment in which the molecules adhering to the column become partitioned into the mobile phase and will move through the column. This can be accomplished by a change in pH, ionic strength, or polarity of the buffer.

Anion exchange chromatography separates based on the negative charge of a molecule. The column is made up of positively charged beads which attract negatively charged molecules flowing through the column. Positive or neutral molecules will pass quickly through the column with little or no interaction with the stationary phase, while negative molecules will attach to the column. The buffers used as the mobile phase in anion exchange chromatography are salts which, at low concentrations, will not affect the

¹⁸ Itakura, Keiichi, John L. Rossi & R. Bruce Wallace *Ann Rev Biochem* **53**, (1984) p 323-356

interaction between the negatively charged molecules and the column. However, by increasing the salt concentration, the salt ions will preferentially bind to the column, masking the positive charge and an exchange will take place. The salt ions bind to the column and the negatively charged molecules elute from the column. This method is used to separate DNA molecules because DNA is polyanionic. Longer oligos will have a higher net negative charge, therefore having a stronger affinity for the column and will elute later than shorter oligos. In this way, the oligos will, in a sense, be eluted by size. Since anion exchange chromatography uses salts, the oligo must also be desalted by dialysis before use in cell studies¹⁸. Anion exchange chromatography, however, has been shown to have poor resolution. It also cannot be used to distinguish between different base sequences of the same length since net charge only depends on length and not base composition^{19,20}.

Reverse phase high performance liquid chromatography (RP-HPLC) is the third method used for oligo purification. This type of chromatography is based on the polarity of molecules. High performance liquid chromatography uses a pump system to create a high pressure that pushes a solvent and sample through a column. It is called reverse phase chromatography because the mobile phase is a polar solvent and the column is non-polar, the reverse of normal phase chromatography. The sample is separated by its partitioning between the mobile phase and the stationary phase. In this case, the molecules that are more non-polar will adsorb to the column while the more polar molecules will readily pass through the column. The polarity of the mobile phase is changed to solvate and elute the non-polar molecules.

¹⁹ Bjergaard, Kirsten & Otto Dahl. *Nucleic Acids Res* **19**, 21 (1991) p 5843-5850

Since the hydrophobicity of an oligo can change with length and base composition, RP-HPLC can distinguish between different DNA base sequences as well as between different lengths. It is also much easier to collect the purified DNA in comparison to gel electrophoresis and requires no desalting prior to use in cell studies. However, this method is only useful for the purification of short to medium length oligomers (less than 25 bases)¹⁶.

Typically in reverse phase, the crude oligo sample is injected into the HPLC and is carried into the column by the mobile phase. The crude oligo contains by-products of the synthesis and detritylated failure sequences which are the capped sequences that are not bound to dimethoxytrityl. It also contains the tritylated sequences which are bound to the dimethoxytrityl group. Separation of the oligos occurs in the column whereby the detritylated failure sequences and by-products of synthesis are separated from the tritylated product peak. Since the dimethoxytrityl group gives the oligo a non-polar character, the failure sequences will be more soluble in the polar mobile phase while the tritylated oligo will be retained by the column. After the polar sequences have eluted from the column, the polarity of the mobile phase is changed so that the tritylated sequences will dissolve and be eluted from the column (see figure 9).

²⁰ Liautard, J., C Ferraz, J Sri Widada, J P Capony & J P Liautard *J. Of Chrom* **476** (1989) p 439-443

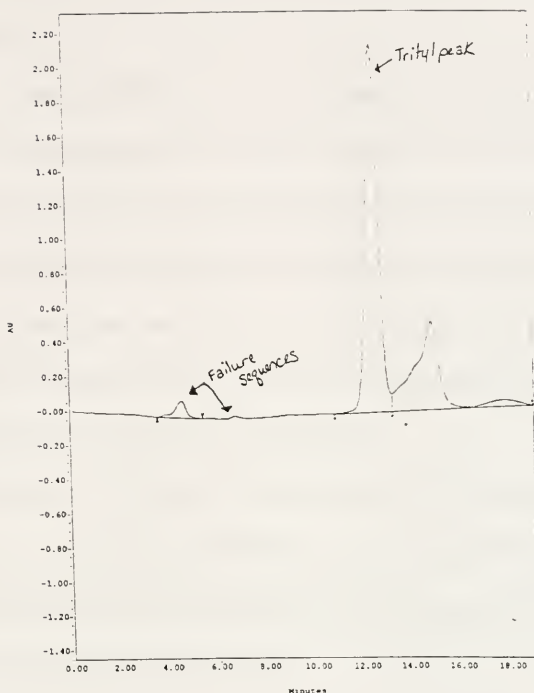


Figure 9: Sample chromatogram of a PO2 oligo separated on RP-HPLC Waters 510 pump system with a triethylammonium bicarbonate (TEAB) buffer and acetonitrile (ACN) gradient, pH 8.20: start at 1mL/min 80%TEAB/20%ACN and switch to 1.5mL/min over 1 min, switch to 60%TEAB/40%ACN over 6 min, switch to 1mL/min over 1 min, hold for 8 min, switch to 80%TEAB/20%ACN over 2 min, hold for 1 min

Both anion exchange chromatography and reverse phase high performance liquid chromatography are fast methods for the purification of oligos and both are useful depending upon the technique needed. Anion exchange chromatography is useful for the separation of oligos that differ in length and has been demonstrated to separate based on base composition although reports are conflicting^{20,21}. RP-HPLC is useful for the

²¹ Meteleev, Valeri & Sudhir Agrawal. *Anal Biochem* 200 (1992) p.342-346

separation of tritylated oligos and results in a much purer product than anion exchange chromatography²².

While the use of synthetic oligos has been employed in antisense drug therapy for many years, only the purification of the normal, unmodified DNA (PO2) and monothiophosphate modified DNA (POS) have been widely published. Much of the research cited in the literature uses anion exchange chromatography and does not provide much in-depth study of these purification procedures using spectral analysis, pH, instrumentation, or temperature. Most of the analysis has been focused on the effect of pH and temperature on the resolution of the separation.

Yoshio Kato, in a 1988 paper, studied the effects of pH, type of salt in eluent, and temperature on the separation of PO2 oligos using an anion exchange column. The pH study (shown in table 1) served to determine the pH at which there is a good resolution in the separation of the oligomers.

pH Range	Observation
4.5-7.5	Tailing on peak, low recovery of PO2
8.5-10.5	Good resolution
8.5-9.5	Better separation based on length of oligo
10.5	Better separation based on base composition

Table 1: Results of pH study run on a DEAE column Gradient: linear gradient of NaCl in 20mM Tris-HCl buffer (pH 9.0) or 1,3 diaminopropane-HCl buffer (pH 10.5) at a temperature of 25°C and flow rate of 1.5mL/min²³

²² Hill, Tonya L. & James W. Mayhew. *J. Of Chrom.* **512** (1990) p 415-431

The effect of temperature on the separation and resolution of the peaks was also studied using a temperature range of 25°C through 65°C. The results showed that the resolution of the peaks were the same for the range of temperatures from 25°C to 45°C, but was reduced at temperatures above this range.

A comparison between two salts, sodium chloride and sodium perchlorate, was also performed. The salts were studied to determine their ability to separate a mixture of oligos based on chain length and a mixture of oligos based on base composition. This analysis concluded that the type of salt used depended upon the type of separation needed because each salt worked better for the separation of one of these mixtures, but not both²³.

In a study of the separation of PO2 oligos using RP-HPLC, Tonya Hill made a comparison among three columns, varying the chain length and pore size of each column (see table 2). These variables were used to determine the best separation method for tritylated oligos of 16 to 100 bases²¹.

²³ Kato, Yoshio, Takashi Kitamura, akane Mitsui, Yosuke Yamasake & Tsutomu Hashimoto. *J. Of Chrom.* 447 (1988) p 212-220

Column Characteristics	C ₈ or C ₁₈ column 80Å	C ₃ column 300Å	C ₈ column 300Å
Length of oligo to be purified	< 25 bases	> 25 bases	> 25 bases
Speed	Moderate strength mobile phase	Fast purification: 5 min	
Gradient	Shallow with isocratic delays		Long, slow gradient

Table 2: Study of effect of column using 0.1M TEAA (pH7.0) as buffer A and 0.1M TEAA in 70% Acetonitrile as buffer B. Gradient for tritylated oligos < 25 bases: 3 min at 15% B, increase to 33% B over 11 min, 33% B for 29 min, increase to 100% B over 4 min at flow rate of 1mL/min. Gradient for tritylated oligos > 25 bases: increase from 15% B to 29% B over 10 min, hold for 20 min, increase to 100% B over 4 min at flow rate of 1mL/min²¹

Warren did a study on the effect of two temperatures, 28°C and 60°C, on the resolution of the separation of a mixture of polyadenylate oligos of differing chain length on an anion exchange column. The results show that for a PO2 oligo, the resolution and separation of peaks was better at 60°C although the peaks did broaden slightly because of the increased salt concentration of the buffers needed¹⁶.

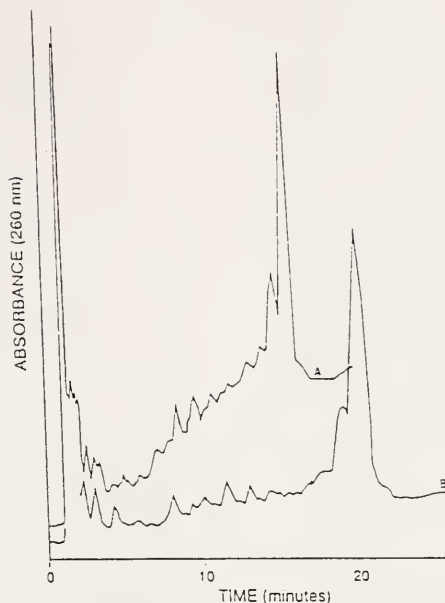


Figure 10: Chromatograms run on 2.5 μ g polyadenylate mixture of 19-30 bases using a high performance anion exchange column at two temperatures (28°C and 60°C) Buffer A: 25mM Tris/Cl, 1mM EDTA pH 8.5. Buffer B: buffer A + 1.0M NaCl. Gradient at 28°C: 10% B to 30% B in 5 min, increase to 40% B in 30 min at flow rate of 1mL/min. Gradient at 60°C: 30% B to 37% B in 5 min, increase to 47% B in 30 min at flow rate of 1 mL/min.

In a temperature study of the separation of DNA restriction fragments on an ion pair reverse phase HPLC, Huber used a temperature range of 20°C to 70°C. The separation of the peaks kept getting better as the temperature increased (see figure 11). The best resolution of peaks occurs at 50°C, at temperatures higher than this the resolution decreases rapidly due to denaturation of the DNA fragments²⁴.

²⁴ Huber, Christian G.; Peter J. Oefner; Eugen Preuss & Günther K. Bonn. *Nucleic Acids Res* 21, 5 (1993) p.1061-1066

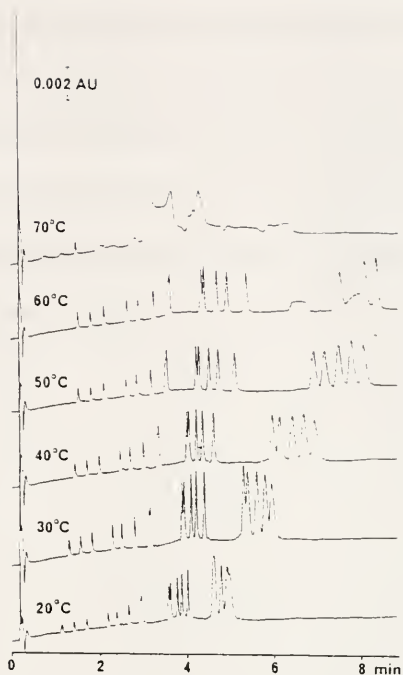


Figure 11: Effect of column temperature on separation of pBR322 DNA digested with 0.5 μ g HaeIII run on Ion-pair Reverse phase polystyrene divinylbenzene C₁₈ column. Mobile phase: 0.1M TEAA (7.0) Gradient: 7.5%-13.75% Acetonitrile (ACN) in 4 min, 13.75%-16.25% ACN in 6 min at flow rate of 1mL/min and detected at 254nm²⁴

These results show that the DNA seems to react more strongly with the column at higher temperatures. The dimethoxytrityl group is held tighter by the stationary phase and a more non-polar solution is needed to elute the DNA from the column. In this way the separation of the peaks is greater²⁴.

However, since PO2 analogs are quickly degraded by nucleases within the cell, studies should focus on more promising modifications of the DNA. One of these modifications is the thio backbone modification, POS. These oligos have been

extensively studied in cell cultures to determine their effect on cancer cells, but there have been few spectral, pH, buffer, or instrument studies on POS oligos to determine the best purification method and obtain the purest peak for the cell studies.

In a comparative analysis between PO2 DNA and POS DNA by Metelev and Agrawal, it was shown that POS oligos are retained by the column longer than the PO2 oligos^{21,25}. These results, as shown in figure 12, show that POS oligos have a higher hydrophobicity than PO2 oligos.

²⁵ Agrawal, Sudhir *Proc. Natl. Acad. Sci. USA* **94** (1997) p 2620-25

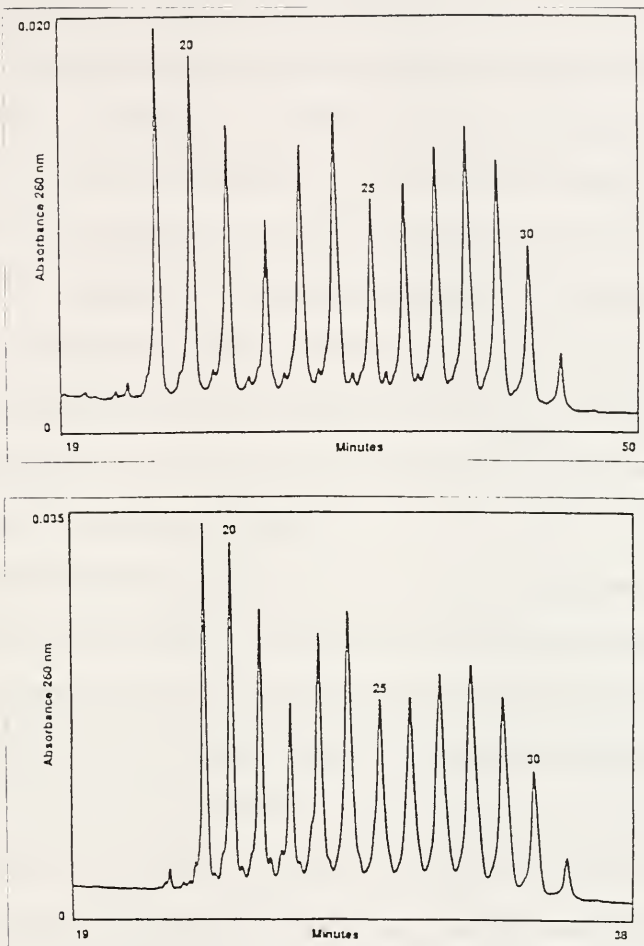


Figure 12: Chromatogram of a PO2 (A) and a POS (B) showing the difference in retention time on a weak anion exchange column. Buffer A: 20mM KH_2PO_4 (pH6.3), 50mM $(\text{NH}_4)_2\text{SO}_4$ /Acetonitrile (75/25). Buffer B: 20mM KH_2PO_4 (pH6.3), 500 mM $(\text{NH}_4)_2\text{SO}_4$ /Acetonitrile (75/25). Flow rate: 1.5mL/min. Gradient: 0%B 100% A for 2 min, increase to 100% B over 30 min. $T_R(\text{PO}_2)$: 16 min. $T_R(\text{POS})$: 20 min²¹.

The POS peaks are also broader than the PO2 peaks which is attributed to the fact that phosphorothioates are chiral and therefore diastereomers occur in synthesis^{21,24}. In a POS oligo the phosphate backbone between each base in the oligo can be in one of two

states, thio modified or oxidized. Most of the POS analogs used in the literature studies and in this research are modified at every base, therefore a POS oligo with one or more oxidized bases is a diastereomer. The number of diastereomers that can occur is a function of the number of modified bases: 2^n where n stands for the number of thio modified linkages in the oligo. For example, a 15 base oligomer has 14 linkages and would have 2^{14} diastereomers. Diastereomers are a problem in the purification of POS oligos because the most biologically active diastereomer is usually unknown and would be difficult to separate from the other diastereomers even if known¹⁴. When the diastereomers remain mixed, they can also create a problem during cell culture by decreasing the effectiveness of the oligo¹³.

Since dithiophosphate modified DNA (PS2) is a recent modification in oligonucleotide structure, little has been published involving purification analyses. A paper by Bjergarde describes the purification of PS2 DNA using RP-HPLC. Purification of the PS2 modified oligo was attempted using anion exchange chromatography using a Pharmacia Mono Q column and RP-HPLC using a C_{18} column. However, these chromatograms resulted in an elevated baseline with no distinct band because the PS2 was too strongly bound to the material in the column. This problem was rectified by separating the PS2 on a Hamilton PRP-1 reverse phase column. The Hamilton PRP-1 column is made of a polystyrene divinylbenzene packing material while the C_{18} column is made of a silica based packing material. Using the PRP-1 column, the oligo is eluted in a single strong peak after 25-30 minutes with very little tailing, although some POS impurities do exist in the collected fraction¹⁹.

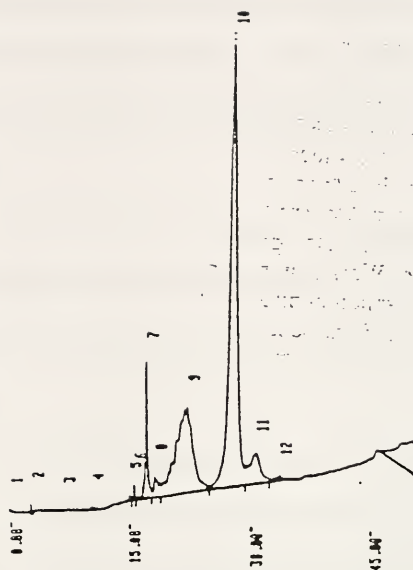


Figure 13: Chromatogram of PS2 17mer (every base modified) with a retention time of 28.3 minutes (peak 10) Column: PRP-1 Buffer A: 5% Acetonitrile in 0.1M TEAB (pH9.0) Buffer B: 80% acetonitrile in 0.1M triethylammonium bicarbonate (TEAB) pH9.0 Gradient: hold 100% A for 5 min, linear increase to 100% B over 40 min, hold 100% B for 5 min.

RESEARCH GOALS

Since the advent of antisense drug therapy, many different modifications have been studied in vivo and in vitro as to their efficiency at inhibiting the growth of cancer, HIV, and other diseases. The effort of most researchers has been on the antisense study of the oligos, while an in depth study of the purification of oligo has fallen into the

background. Since the purification process leads to a pure oligo that can be used in cell studies, a study of the purification procedure must be completed in order to increase the yield and produce the purest oligo possible. Continued analysis of the purification of oligos, using these preliminary studies as a guide, could lead to a more time and cost effective method.

The overall goal of this research project is to study all aspects of antisense therapy, specifically focusing on the PS2 modification. This objective developed into a multi-tiered project, focusing on the synthesis, purification, and biological effect of the antisense oligos.

Five different modified oligos have been used as models; including PO2, POS, and PS2 oligos. Three different PS2 oligos were used each containing one, two, or three dithio modifications. One of these modified oligos has a dithio modification in the middle of the base sequence(PS21), the second modified oligo has a dithio modification at each end (PS22), and the third modified oligo has a dithio modification in the middle and at each end of the base sequence (PS23). Figure 14 gives the sequence and dithio modification positions for an antisense oligo directed against the Rous sarcoma virus (RSV).

PS21: 5'-T_p G_p A_p A_p T_p G_p G_p T_x A_p A_p A_p A_p T_p G_p G-3'

PS22: 5'-T_x G_p A_p A_p T_p G_p G_p T_p A_p A_p A_p A_p T_x G_p G-3'

PS23: 5'-T_x G_p A_p A_p T_p G_p G_p T_x A_p A_p A_p A_p T_x G_p G-3'

Figure 14: Anti-RSV sequences demonstrating different PS2 modifications (x is a PS2 substitution, p is a PO2 linkage)

The synthesis component of the project deals mainly with the analysis of the synthesis of monothiophosphoramidite and PS2 modified oligos to ultimately increase the yield and ease of synthesis. The synthesis of the monothiophosphoramidite, worked on by Jennifer Swisher, Kristy Winstead, and Rebekah Cowell, requires the synthesis of two precursor molecules, ethanedithiol monobenzoate and tris(pyrrolidino) phosphine. All of these syntheses are long (13-24 hours) and are air and water sensitive. Therefore increasing the yield of all these syntheses would be very beneficial in saving both time and money. The analysis of the synthesis of the PS2 modified oligos on the DNA synthesizer is now being studied by Kristin Smith to determine the location of loss during synthesis and to eliminate that loss.

The biological aspect of the project involves cell studies of the effect of the antisense oligos and have been conducted by Leslie Farinas, Brandi Whitley, Kristy Winstead, and Heather Thomas. Cell studies were performed to determine the anti-oncogenic effect of the *myc* oligo on HL60 human leukemia cells and to determine whether a specific oligo sequence effect is involved. The *myc* oncogene is responsible for cell growth; therefore, growth and viability of the cells was observed over time. In this study, anti-*myc* PO2, POS, PS21, PS22, and PS23 were added separately to a suspension HL60 of human leukemia transformed cells. Lipofectin is used to aid in oligo uptake into the cells. Control experiments consisted of a suspension of HL60 human leukemia cells alone, with lipofectin added, and a third had a POS control sequence added. The POS control sequence consists of the same base composition in a scrambled order. Every 12 hours, aliquots of the suspensions were removed and the number of viable cells were counted.

As shown in the graph (see figure 15), the cells alone grew normally while cells treated with lipofectin had no growth rate. This may be due to the fact that the lipofectin disrupts the cell in some way. The cells treated with PO2 shows an initial decline in the growth of the cells, but the cells start to recover after approximately 48 hours. This suggests that the PO2 initially interferes with the oncogene but is then degraded and the cells recover. The POS has a greater effect on the cells, as expected, but it also only interferes temporarily before being degraded. The PS2 oligo is not degraded by endonucleases so there is no recovery at a later time.

time (hrs)	alone	lipo 5ul	PO2	POS	POSC	PS2-1	PS2-2	PS2-3
0	115	115	115	115	115	115	115	115
12	112	100	86	84	82	78	72	63
24	120	98	84	80	78	75	70	58
36	122	97	83	79	77	72	69	45
48	124	96	82	78	77	68	68	46
60	126	94	84	77	75	67	66	43
72	128	92	85	77	73	66	62	38
84	129	89	87	75	68	65	58	36
96	130	88	88	73	60	64	56	35
108	133	86	89	69	59	60	55	33
120	134	84	94	68	58	57	55	30
132	135	80	94	66	56	55	50	29
144	137	79	95	64	55	50	48	28

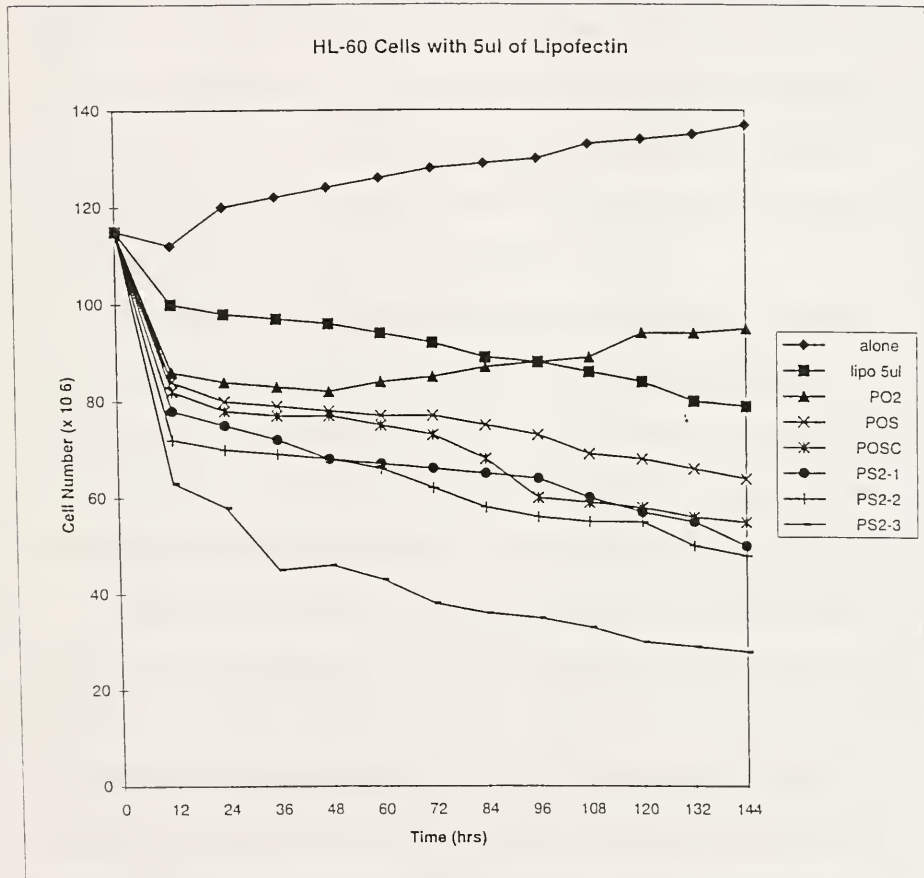


Figure 15: Graph of anti-myc oligos effect on human leukemia cell growth over a 144 hour period²⁶

²⁶ Farinas, Leslie Independent Study Research Paper Sweet Briar College Spring 1997.

Although all of the PS2 DNA is expected to have a greater effect on the growth of the transformed cells than the PO2 or POS DNA, the PS22 and PS23 seem to have a greater effect than the PS21 oligo. This may be due to the sulfur modifications on the ends of the PS22 and PS23 sequences causing increased endonuclease resistance.

As part of this multi-tiered project, the analysis of the purification of oligos to determine a method that gives the purest oligo is the main goal of this thesis project. The purification of the PS2 oligos will especially be examined as they are the least studied in the literature. The overall goal of determining a purification method is advanced in several ways: first by a spectral study, second by a pH/buffer comparison, third by an instrument comparison, and lastly by a temperature comparison.

The spectral study of the RP-HPLC chromatogram determines the Waters RP-HPLC 510 and 626 pump systems with a Waters 996 PDA detector ability using Millennium 2010 Chromatography Manager Version 2.15 to distinguish among the PO2, POS, and PS2 forms of DNA using the spectra alone. Using a spectral library, a library match and purity match can be calculated for each modified oligo being tested.

When performing a library match, the program calculates and reports a match angle and a match threshold based on the comparison between the library spectra and the spectra to be matched. The Millennium program compares the spectra at the apex of the peak to the library through the use of vectors. To accomplish this, the ratio of the absorbance of the spectra at two different wavelengths is determined and translated into a vector. The same is done for the spectra to be matched and the vectors are plotted on a

graph of absorbance at the first wavelength versus absorbance at the second wavelength.

The angle between the two vectors is called the match angle.

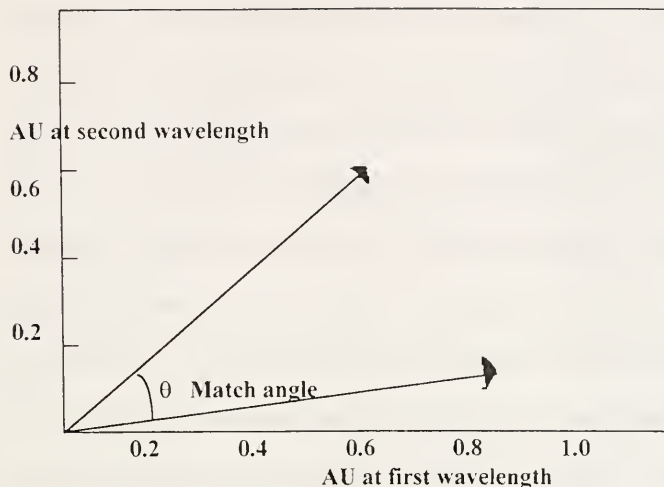


Figure 16: Example of plotting vectors from two match angles to calculate the match angle

The match threshold is the largest match angle that could be due to noise or solvent, but not to an actual difference in spectral shape. Ideally, the match angle would be zero when matched against the same oligo. However, even with an automated system, the conditions and parameters will not be exactly the same for each run; a slight difference in background noise, pH, or solvent composition would have an effect on the spectral shape. Therefore an ideal match would have a match angle lower than the match threshold²⁷.

²⁷ Waters PDA Software Users Guide

Determination of the peak purity of the main product peak in the chromatogram indicates the number of impurities present and helps to compare the separation of each peak. The presence of spectrally distinct elements in the peak are detected by comparing the spectra within the peak to the spectrum at the peak apex. The purity angle is calculated by taking the average of the comparison of each spectra within the peak to the spectra at the peak apex. The purity threshold is calculated the same as the library match. If the purity angle is greater than the purity threshold, then the peak is impure. The number of impurities is indicated by the number of purity passes it takes before the purity angle is less than the purity threshold. A purity pass is one calculation of the purity angle and threshold. The subsequent purity passes uses the maximum impurity spectra from the previous purity passes to recalculate the purity angle and threshold. The maximum number of purity passes allowed by the Millennium software is four, so the maximum amount of coelutions that can be detected is three²⁷.

Purity Pass	Purity Angle	Purity Threshold	Location of Maximum Impurity (min)
1	1.807	1.732	13.265
2	0.681	1.740	12.673
3	0.425	1.741	13.707
4	0.172	1.745	13.048

Table 3: Example of a purity/ match table showing one impurity in the peak

There are three types of coelutions that can occur: front, tail, or intermediate coelutions. By combining the information for the library match results and the purity match results, the type of coelution can be determined. This is because the library match

results are more sensitive to front or tail coelutions but cannot detect intermediate coelutions while purity match results can detect intermediate coelutions but not front or tail coelutions²⁷.

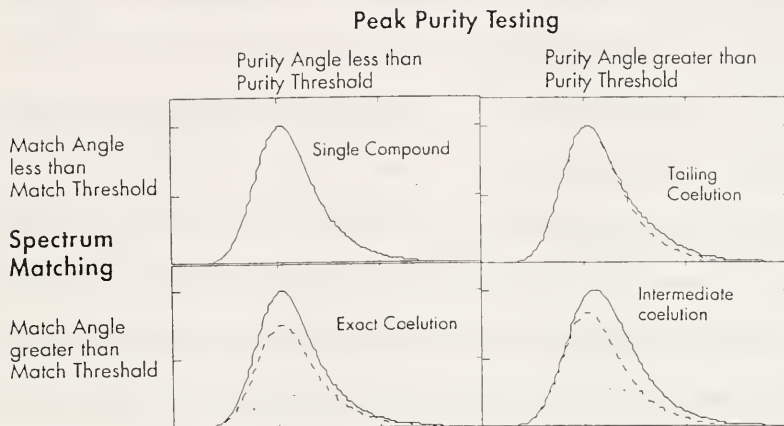


Figure 17: Determination of type of coelution from library and purity information²⁷

There have been no reports in the literature studying this facet of oligonucleotide purification and, if successful, these spectral studies will help the researcher evaluate each purification method and will aid in maximizing the purification of each modified oligo in an effort to achieve a pure oligo for use in antisense therapy.

The second goal of this thesis research is a combined pH study and buffer comparison of the mobile phase used for RP-HPLC of all five modified oligos. As mentioned previously, there are reports in the literature on pH studies, but they were done on an anion exchange column and only with PO2 analogs. Reports in the literature also

show that the purification methods which involve RP-HPLC primarily use one of two buffers, triethylammonium acetate (TEAA) and triethylammonium bicarbonate (TEAB) at a pH range of 7.0 to 9.0, but no comparison has been made^{22,25,28}.

The processing methods developed in the spectral study will be used to determine the purity of the product peak for each modification at each pH and any improvement in the spectra or resolution of the chromatogram peak. These criteria will be used to establish the best pH and buffer at which to purify PS2 modified oligos.

The third goal of this thesis research is an instrument comparison to examine the difference between the new low dispersion, low volume pump system (Waters 626) and the traditional pump system (Waters 510). The comparison of these two pumps is done because the 626 pumping system is a polymeric system and is advertised to be more biocompatible than the 510 pumping system. In theory, the polymeric system eliminates interaction of the DNA with the metal, which occurs in the all stainless steel pumps and tubing of the 510 pumping system. Specifically, the sulfur modification is theorized to interact more with the metal contained in the 510 pumps than the low volume, polymeric 626 pump system. Therefore the 626 pump system should maximize the yield and resolution of the separation of the POS and PS2 oligos. The spectra extracted at 254nm will also be analyzed to ascertain whether the 626 pump provides better resolution and purity of the oligo peak, especially with the PS2 modifications, than the 510 pump.

The final goal is a study of the effect of column temperature on the separation and spectra of PO2, POS, and PS21, PS22, PS23 modifications. There are a variety of temperatures cited in the literature ranging from 25°C through 60°C, but there are few

²⁸ Lawson, T G , F E. Regnier, & H L. Weith *Anal. Biochem.* **133** (1983) p.85-93

studies about the effect of temperature upon the purification of oligodeoxynucleotides, and none for PS2 modified oligos. The data will be processed as in the previous studies and analysis of the purity profile, improvements in spectra, chromatogram profile, and yield of oligo will be used to determine the best temperature for the purification of each modification.

EXPERIMENTAL METHOD

The oligos used in this study were comprised of a 15 base sequence, 5'-AAC GTT GAG GGG CAT-3', complementary to the *myc* oncogene and a 15 base sequence, 5'-TGA ATG GTA AAA TGG-3', complementary to Rous Sarcoma Virus (RSV). All the monothiophosphate modified oligos used were modified at each base linkage. The first anti-*myc* POS control sequence was made incorrectly so it has a different base composition from the anti-*myc* POS; the control contains an extra G and has one less C than the POS. The dithiophosphate modified oligos used were modified in one, two, or three base linkages as described previously (see figure 14). All the oligos used were synthesized using a Pharmacia LKB Gene Assembler Special using deoxynucleoside phosphoramidites, tetrazole, acetonitrile, 1,2 dichloroethane, detritylation solution, capping A and B solutions, Beaucage reagent, and supports purchased from Pharmacia.

Purification of all oligos was done by RP-HPLC using a Hamilton PRP-1 (polystyrene divinylbenzene) column. A Waters 510 tertiary pump system connected to a Waters 996 PDA detector was used for the pH/buffer study and the instrument

comparison. A Waters 626 quaternary pump system connected to a Waters 600S controller and the same Waters 996 PDA detector was used for the temperature study and instrument comparison. Data acquisition and analysis were performed using Millennium 2010 software version 2.15 on a 486DX2 66Mz IBM compatible computer. Solvents used were HPLC grade acetonitrile and methanol purchased from Sigma, 0.1M triethylammonium acetate buffer (TEAA), and 0.050M triethylammonium bicarbonate buffer (TEAB) made in the lab with pure, distilled water from a Modulab Modupure Plus.

The DNA spectral library was built using the Waters 510 tertiary pump system. This library consists of a spectrum from each of the five modifications for sequences of anti-RSV and anti-*myc*. In order to match spectra to this library, a value for the noise and solvent threshold is determined and used in the processing parameters. This value is based on the types of solvents used in the gradient and the noise generated by the Waters 996 PDA detector in the baseline of the chromatogram. To determine this value, six replicate runs on the RP-HPLC of PO2 oligos were analyzed. The highest match and purity angles from these runs is entered into the processing method to be used in subsequent spectral analysis.

For all of these studies, the PDA detector records spectra at a rate of 2.0 spectra/sec over a wavelength range of 210 to 400 nm. The resolution for each chromatogram is set at 2.4 nm which stands for the number of diodes of the detector averaged as a single spectral data point (each diode is 1.2 nm). This small resolution number is used to obtain greater spectral resolution in order to differentiate between the closely related spectra of each modification. A chromatograph is then extracted at a

wavelength of 254 nm and the peaks are integrated by a programmed processing method. The integration of the peaks finds the retention time at the lambda max and extracts a spectrum for each peak.

The pH/buffer study was performed on the 510 pump system using duplicate runs of PO2, POS, and PS23 at pH of 7.0(± 0.1), 7.4(± 0.1), 7.8(± 0.1), 8.1(± 0.1) with TEAA buffer. A preliminary study comparing the effect of TEAB buffer on the spectra of PO2 and POS at pH of 8.1(± 0.1) was also performed. The pH was monitored using an Orion Research model 601A\digital IONALYZER.

The preliminary temperature study was run on the 626 pump system which includes a column heater. The PS2 modification, PS21, and the spectral library was used to observe whether the purity and separation were affected at temperatures of 25°C, 30°C, and 37°C.

The preliminary instrument study focused on the PS2 modifications, specifically PS21 and PS22, to observe the effect on purity and separation. Each modification was run using the same 80 minute acetonitrile gradient with TEAA on both the Waters 510 and 626 pump systems.

RESULTS AND DISCUSSION

The typical chromatogram of synthetic oligos protected by DMTr is identified by the early elution of failure sequences followed by the elution of tritylated product peak. Occasionally another peak follows the product peak and is only identified as the high

eluting organic²¹. The retention time and peak width of the tritylated peak of each chromatogram can give information about the separation of the oligo. When the oligos are run with the same gradient, the retention times of the PS2 modifications are the longest, followed by the POS oligos, and then the PO2 oligos. Although all of the oligos contain a hydrophobic dimethoxytrityl group, the dithio modifications further increase the hydrophobic nature of the oligo so that the PS2 oligos are retained by the column longer. The POS modification is slightly less hydrophobic than the PS2 modifications and therefore it doesn't interact with the stationary phase as long. The PO2 oligo is only hydrophobic due to the dimethoxytrityl group and therefore has the lowest interaction time of the three modifications studied.

Retention time is, however, affected by the gradient used. The gradient is used to separate the peaks while maintaining a reasonable run time. To accomplish this, the gradient needs to be changed throughout the run to change the eluent conditions in the column. For this project, the gradient is changed by increasing or decreasing the amount of acetonitrile. When the amount of acetonitrile is low, the failure sequences quickly elute from the column and the tritylated oligos partition with the stationary phase. As the amount of acetonitrile increases, the solvent becomes more hydrophobic and the tritylated oligos are eluted from the column. Changing the flow rate also affects the separation of the peaks as well as the peak width half-height. A higher flow rate (1.5mL/min) results in a quicker elution and pushes the peaks together. Therefore, this technique is used to quickly elute the failure sequences. A slower flow rate (0.75mL/min) slows down the elution and increases the separation between the peaks. However, a slow flow rate also broadens the peaks and dilutes the concentration of the

oligo. An ideal gradient quickly elutes the failure sequences, separates the failure sequences and tritylated oligos well, and retains a sharp, narrow product peak. Figure 18 shows an ideal gradient of the separation of the PO2 oligo. The failure sequences are eluted in under 6 minutes and are well separated from the tritylated peak which elutes in a sharp peak and has a peak width at half-height (PWHH) of less than one minute. This means that there is a good, undiluted separation of the DNA sequences. As the PWHH gets larger, the product is diluted more by the solvents, so a smaller PWHH is desired.

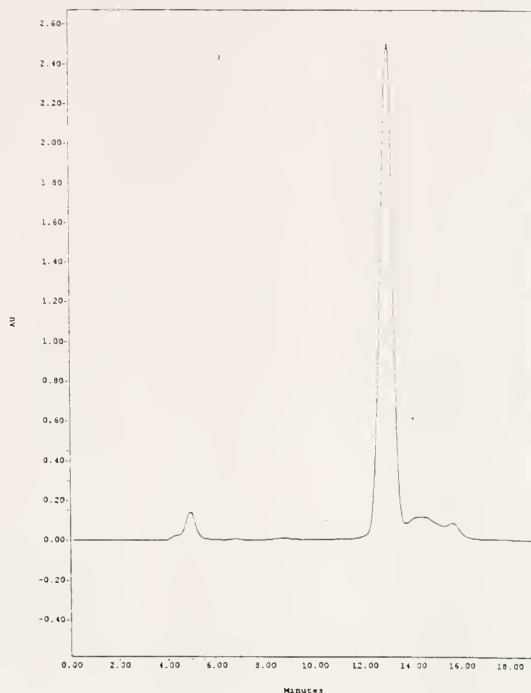


Figure 18: Chromatogram of PO2 oligo run on PRP-1 column of Waters 510 pump system with $T_R=12.889$ min Gradient, pH 7.98: start at 1mL/min 80%TEAA/20%ACN and switch to 1.5mL/min over 1 min, switch to 60%TEAA/40%ACN over 6 min, switch to 1mL/min over 1 min, hold for 8 min, switch to 80%TEAA/20%ACN over 2 min, hold for 1 min

The gradient used for the separation of POS (see figure 19) is ideal for the quick elution of failure sequences, but the tritylated peak has a PWHH of approximately 4 minutes which is far too broad. Therefore the gradient needs to be lengthened at that point to allow further separation of the shoulder peak and the tritylated peak.

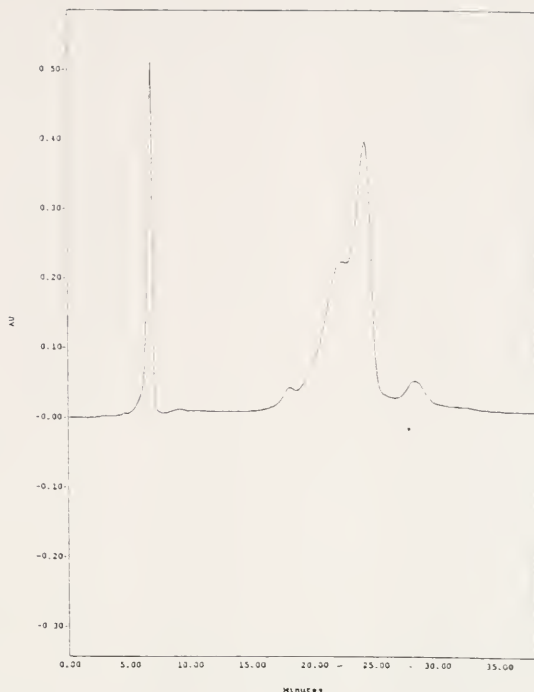


Figure 19: Chromatogram of POS oligo run on PRP-1 column of Waters 510 pump system with $T_R=23.960$ min Gradient, pH 8.22: start at 1mL/min 80%TEAA/20%ACN and switch to 1.5mL/min over 1 min, switch to 70%TEAA/30%ACN over 6 min, switch to 1mL/min over 1 min, hold for 5 min, switch to 60%TEAA/40%ACN over 7 min, hold for 15 min, switch to 80%TEAA/20%ACN over 2 min, hold for 1 min

The chromatogram of the PS21 oligo (see figure 20) again shows a quick elution of the failure sequences and a good separation and small PWHH of the tritylated oligos.

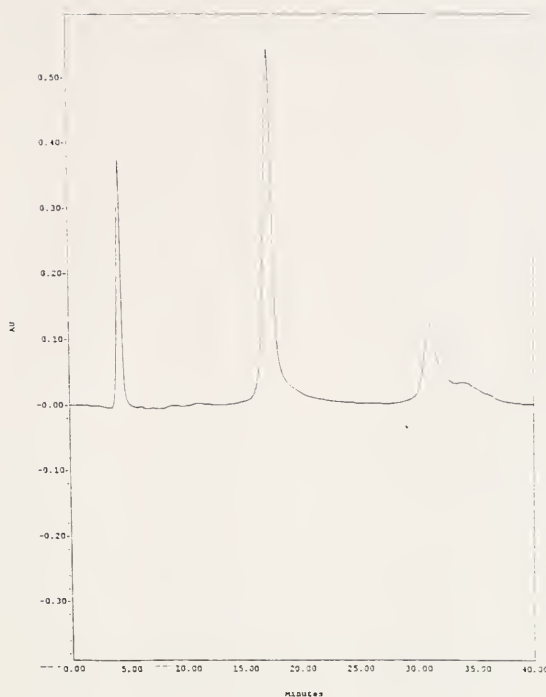


Figure 20: Chromatogram of PS21 oligo run on PRP-1 column of Waters 510 pump system with $T_R=17.286$ min Gradient, pH 8.31: start at 1mL/min 80%TEAA/20%ACN and switch to 1.5mL/min over 1 min, switch to 70%TEAA/30%ACN over 6 min, switch to 1mL/min over 1 min, hold for 10 min, switch to 60%TEAA/40%ACN over 9 min, hold for 10 min, switch to 80%TEAA/20%ACN over 2 min, hold for 1 min

The chromatograms of the PS22 and PS23 are almost exactly the same (see figure 21 and 22). Both have quick elution of the failure sequences, a retention time of about 21 minutes, and a PWHH of 1 minute. Although the PWHH is low, visual inspection shows a shoulder at the tail end of the tritylated peak. Therefore the gradient needs to be lengthened during the elution of the tritylated peak to further separate the shoulder from the product peak.

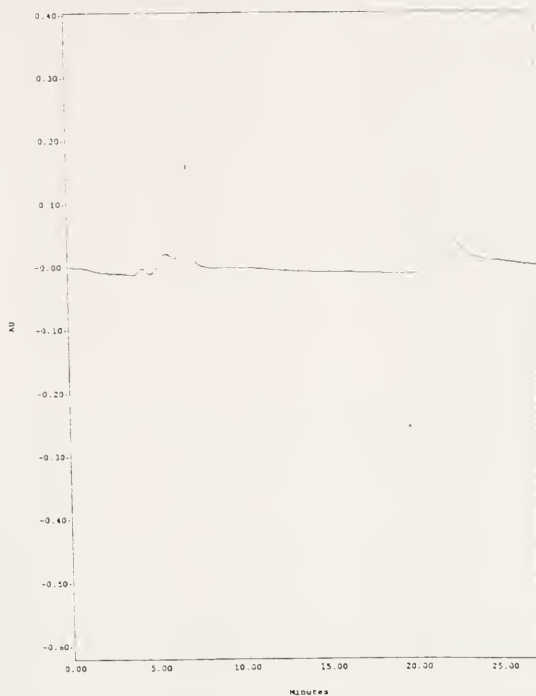


Figure 21: Chromatogram of PS22 oligo run on PRP-1 column of Waters 510 pump system with $T_R=21.098$ min Gradient: start at 1mL/min 80%TEAA/20%ACN and switch to 1.5mL/min over 1 min, hold for 6 min, switch to 0.75mL/min and 60%TEAA/40%ACN over 1 min, hold for 12 min, switch to 80%TEAA/20%ACN over 5 min, hold for 2 min

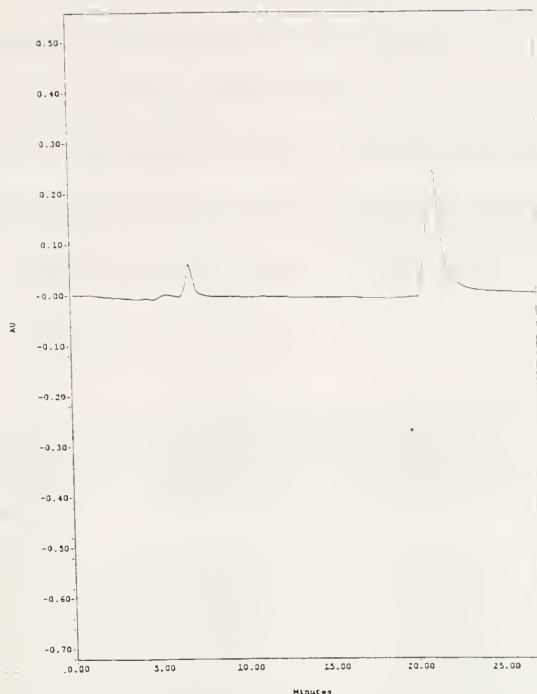


Figure 22: Chromatogram of PS23 oligo run on PRP-1 column of Waters 510 pump system with $T_R=21.030$ min Gradient: start at 1mL/min 80%TEAA/20%ACN and switch to 1.5mL/min over 1 min, hold for 6 min, switch to 0.75mL/min and 60%TEAA/40%ACN over 1 min, hold for 12 min, switch to 80%TEAA/20%ACN over 5 min, hold for 2 min

Spectral Analysis

In the spectral analysis study, representative chromatograms of each modification for both anti-RSV and anti-*myc* oligos were integrated and the spectrum of each tritylated peak were put into a DNA spectral library. The fact that not all of the oligos were run using the same pH or gradient was noted. The spectral library was then tested using spectra from the tritylated peak of the anti-*myc* oligos. First, the PO2 oligo to be matched

was compared with the spectral library and the Millennium software calculated the match angle and match threshold for the match and gave the result in tabular form. The rest of the modified oligos were then matched in the same way.

The library match table below (table 4) is given in the following format, the first column contains the name of oligo sample spectrum to be matched, the second and third columns contain the match angle and match threshold respectively, and the final column contains the name of the library spectrum.

Library Match Results			
DNA Modification (spectrum to be matched)	Match Angle	Match Threshold	Spectrum Match
PO2	0.101	1.610	Tritylated PO2
POS	2.327	1.727	Tritylated POS
PS21	2.648	2.108	Tritylated PS21
PS22	0.930	1.781	Tritylated PS22
PS23	3.779	1.614	Tritylated PS23

Table 4: Table of best library matches for the tritylated peak of each modification using the DNA spectral library

As shown in the above table, the library spectra for each modification is matched correctly by the Millennium software. Visual inspection of the spectra shows the close relation between each modification (see figures 23 and 24), so these results illustrate the program's ability to distinguish among infinitesimal differences in the spectra. However, some of the matches are not ideal as the match angle was greater than the match threshold. This is due to the fact that the oligos within the spectral library were not all run at the same pH, gradient, or concentration as the oligos being matched to the library. The effect of these variables does translate into a difference in spectral shape²⁷.

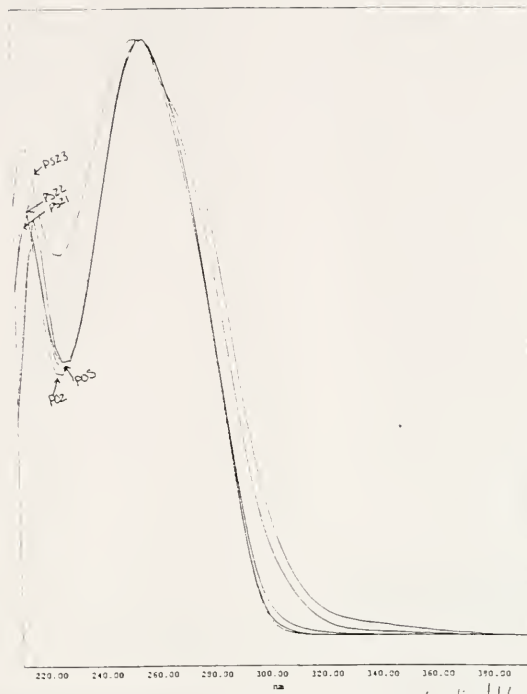


Figure 23: Overlaid spectra of tritylated peaks extracted at 254nm of anti-myc PO2, POS, PS21, PS22, PS23 oligos



Figure 24: Overlaid spectra of tritylated peaks extracted at 254nm of anti-myc PS21, PS22, PS23 oligos

Controversy on the capability of RP-HPLC to separate oligos based on base composition have been reported in the literature^{20,24,29,30}. Liautard states that only anion exchange chromatography is able to distinguish between different base compositions, and that RP-HPLC can only separate oligos based on the presence of the dimethoxytrityl group and does not depend upon the base composition of the sequence²⁰. Zieske states that RP-HPLC is able to separate oligos based on the composition of the oligos³⁰. The

²⁹ Edwardson, P A D, I J. Collins, M D. Scawen, T. Atkinson, H B Cox; S Sivakoff, & R W Stout. *J. Of Chrom.* **545** (1991) p.79-89

results from this research demonstrates that RP-HPLC is able to separate oligos with different base compositions. Furthermore, the library match results also show the ability of the Millennium program to distinguish between the different base compositions of the anti-RSV (5'-TGA ATG GTA AAA TGG-3') and anti-*myc* (5'-AAC GTT GAG GGG CAT-3') sequences. The spectra of the tritylated peaks of both sequences are present in the library. However, when a spectral library match is performed on an anti-*myc* oligo and the match results are reported, no anti-RSV oligos are represented. The match values of nine library matches (out of a total of 15 spectra in the library) for each anti-*myc* oligo were done, but a match to an anti-RSV oligo was not given in these nine results. Therefore, the software does not match an anti-*myc* spectra to an anti-RSV sequence because the spectra are sufficiently different.

Additionally, this capability was also shown by the use of the spectra of an anti-*myc* control sequence matched against the library. The following library match tables are given in the following format. The heading over the table denotes the oligo sample spectrum that was to be matched to the spectral library. The first column contains the match angle calculated by the software. The second column contains the match threshold which is also calculated by the software. The final column, titled spectrum name, contains the name of the library spectrum which matched the sample spectrum.

³⁰ Zieske, L R *Biochromatography* 3, 3 (1988) p.112-117

Library Match Results Anti- <i>myc</i> POS control		
Match Angle	Match Threshold	Spectrum Name
0.874	1.538	Tritylated POS control
2.415	1.544	Tritylated POS

Table 5: Library Match Results from spectra matching of anti-*myc* POS control sequence showing software's capability to distinguish between spectra of compounds with different base compositions

Since the first anti-*myc* POS control sequence, having a G in place of a C, has a slightly different base composition from the anti-*myc* POS sequence, the change in spectral shape should be slightly different and should test the matching capability of the Millennium software. However, the match angles were still significantly different between these two sequences. Although the POS oligo matched the POS control sample, the POS control library spectra was clearly the ideal match for the POS control to be matched since the match angle was less than the match threshold (see table 5). This means that the spectral shape of both the POS control spectra in the library and the spectra to be matched were similar enough that the differences between them could be accounted for by noise or solvent differences during the runs. These results also reinforce the idea that RP-HPLC can separate oligos based on the criteria of different base compositions and this separation is accurate with just one base difference.

One reason the spectra of the anti-*myc* and anti-RSV sequences are different may be due to the numbers of purines or pyrimidines contained in each sequence. Studies have shown that purines are more hydrophobic than pyrimidines and therefore elute slower when using a reverse phase HPLC column²¹. This being the case, then the retention time of the anti-*myc* and anti-RSV oligos should also be different since the anti-

myc sequence contains 10 purines and 4 pyrimidines while the anti-RSV contains 11 purines and 4 pyrimidines. Therefore the anti-RSV sequences should elute later than the anti-*myc* sequences and the differences in the solvent mixture required to elute the anti-*myc* and anti-RSV tritylated peaks would cause a difference discernible to the Millennium software.

Examination of the chromatogram also involved determining the purity of the peak. The purity match results of the spectra used in the library match show the number of impurities in the tritylated peaks of each modification. The format of the purity match tables follow a similar format to the library match result tables. The heading over each set of purity results refers to the oligo being analyzed. The first column is the number of the purity pass done by the Millennium software. The second and third columns are the purity angle and purity threshold, respectively. The final column contains the time at which the maximum impurity within the peak is found during each purity pass.

Purity/Match Results

Anti-myc PO2

Purity Pass	Purity Angle	Purity Threshold	Location of Maximum Impurity (min)
1	1.807	1.732	13.265
2	0.681	1.740	12.673
3	0.425	1.741	13.707
4	0.172	1.745	13.048

Anti-myc POS

Purity Pass	Purity Angle	Purity Threshold	Location of Maximum Impurity (min)
1	1.739	1.872	26.380
2	0.544	1.893	22.797
3	0.342	1.899	24.755
4	0.183	1.902	23.347

Anti-myc PS21

Purity Pass	Purity Angle	Purity Threshold	Location of Maximum Impurity (min)
1	5.052	2.756	17.773
2	1.676	2.871	16.798
3	0.986	2.924	18.198
4	0.825	2.932	20.915

Anti-myc PS22

Purity Pass	Purity Angle	Purity Threshold	Location of Maximum Impurity (min)
1	3.612	1.818	24.647
2	0.682	1.851	20.530
3	0.491	1.847	23.613
4	0.336	1.844	22.913

Anti-myc PS23

Purity Pass	Purity Angle	Purity Threshold	Location of Maximum Impurity (min)
1	2.632	1.699	21.890
2	0.953	1.707	21.407
3	0.294	1.715	20.540
4	0.166	1.707	22.590

Table 6: Results of purity/match results for tritylated peak spectra used in library match (Table 1) of anti-myc PO2, POS, PS21, PS22, PS23 to show any impurities present

The purity passes serve as a type of confidence level for the number of spectrally distinct components within the peak and when the purity angle is above the purity threshold there is coeluting compound. Therefore this data shows that the tritylated peaks of each oligo has only one impurity with the exception of the POS oligo which has no coeluting compounds. The impurities could have a number of origins including tritylated failure sequences or organic by-products from synthesis. In order to make a hypothesis as to the origin and identity of the coeluting compounds, further information on the type of coelution is helpful.

Together, the library match results and the purity match results can be used to determine the location of coelutions, if any are present, using the information given in figure 17.

Coeluting Compounds			
DNA modifications tested	Number of impurities	Spectrum Matching vs Purity Results	Location of Coeluting Compounds
PO2	One	Match angle<Match threshold Purity angle>Purity threshold	Tailing Coelution
POS	None	Match angle<Match threshold Purity angle<Purity threshold	No coelution
PS21	One	Match angle>Match Threshold Purity angle>Purity threshold	Intermediate coelution
PS22	One	Match angle<Match threshold Purity angle>Purity threshold	Tailing coelution
PS23	One	Match angle>Match threshold Purity angle>Purity threshold	Intermediate coelution

Table 7: The locations of each impurity within tritylated peak of anti-myc PO2, POS, PS21, PS22, PS23 oligos using library match and purity/match results with figure 17

The impurities associated with the PS2 oligos may be due to the presence of monothiophosphate impurities although the origin of these impurities is unknown²⁰.

The impurities associated with all of the oligos could also be attributed to the incomplete deprotection after synthesis. If not treated properly with ammonia, some oligos are not properly deprotected. This means that some of the protecting groups on the bases were not completely taken off of the bases. Since these oligos are still tritylated but not fully deprotected, they should still have approximately the same retention times as the fully deprotected tritylated peaks which would account for coelutions.

Instrument Comparison

In the case of the PS21 oligo, the chromatogram appears to show an increase in separation when run using the 626 pump system (see figure 25). By examining the product peak, a more definite separation can be seen between the two peaks present in the 626 chromatogram than in the 510 chromatogram. The PWHH of the tritylated peak separated on the 626 pump is only 1 minute as compared to 2 minutes for the separation on the 510 pump. This means the 626 pump gives a much sharper peak than the separation on the 510 pump. Although, the retention time of the tritylated peak was also much shorter (21.773 min) using the 626 pump than the 510 pump (25.508 min), a good separation of more than 10 minutes from the elution of the failure sequences was maintained. This decrease in retention time is due to the low volume, low dispersion, polymeric 626 pump system. This pump design allows a faster purification of oligos as

well as minimizing the effects of sulfur within the system. Therefore the PS2 oligos are not hung up in the column as could happen in the 510 pump system. From these preliminary results, it is concluded that the use of the 626 pump system should be studied further as to its separation of PS2, POS, and PO2 oligos.

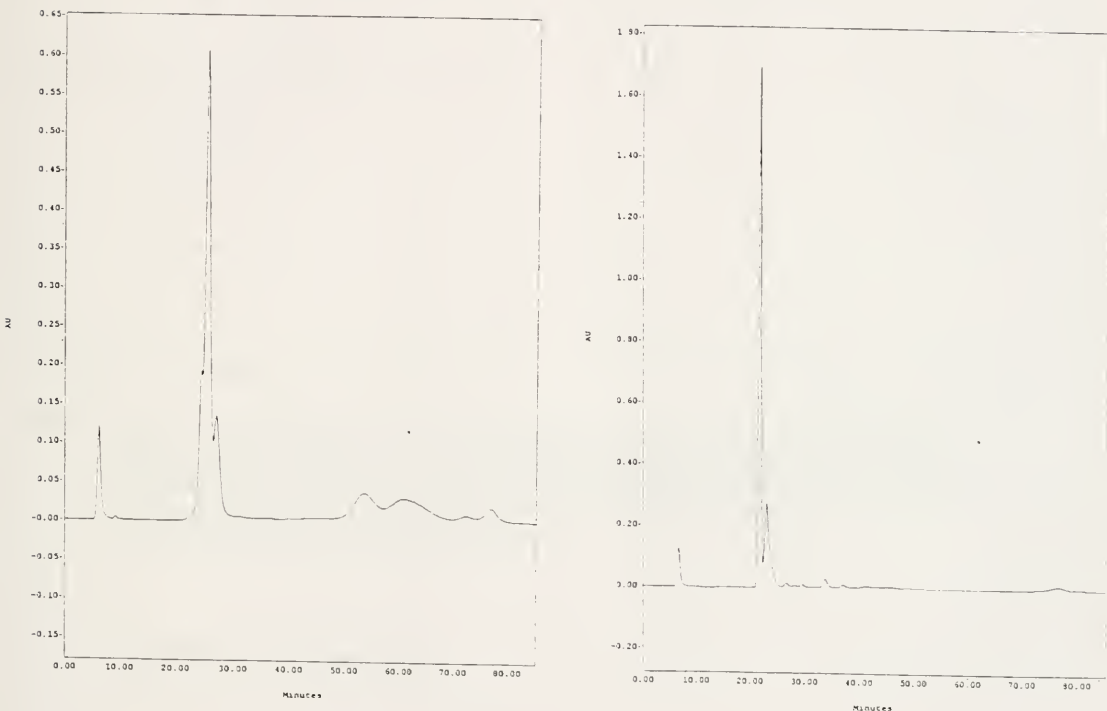


Figure 25: Chromatogram of anti-RSV PS21 using PRP-1 column on (a) 510 pump system and (b) 626 pump system Gradient: start at 1mL/min 80%TEAA/20%ACN and hold for 10 min, switch to 70%TEAA/30%ACN over 3 min, hold for 47 min, switch to 60%TEAA/40%ACN over 10 min, hold for 5 min, switch to 80%TEAA/20%ACN over 5 min, hold for 5 min

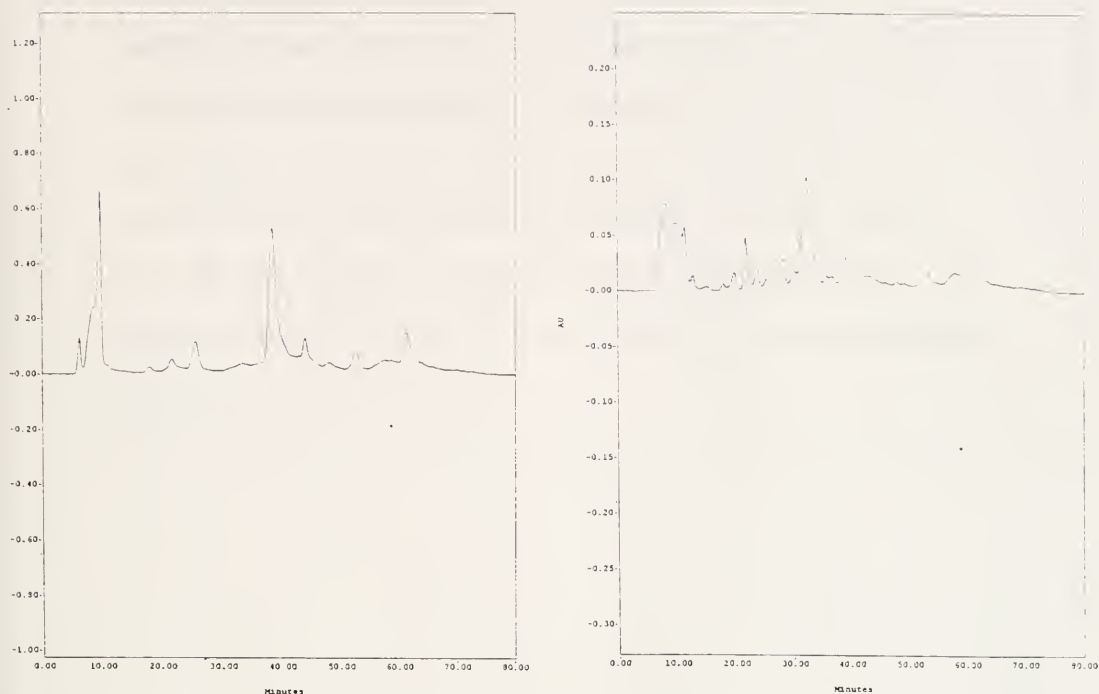


Figure 26: Chromatogram of anti-RSV PS22 using PRP-1 column on (a) 510 pump system and (b) 626 pump system Gradient: start at 1mL/min 80%TEAA/20%ACN and hold for 5 min, switch to 70%TEAA/30%ACN over 10 min, hold for 10 min, switch to 60%TEAA/40%ACN over 10 min, hold for 10 min, switch to 50%TEAA/50%ACN over 10 min, hold for 10 min, switch to 80%TEAA/20%ACN over 10 min, hold for 5 min

pH/Buffer Comparison

The results of the pH study showed that the separation of the oligos increased as the pH of the buffer increased from 7.0 to 7.8. The TEAA buffer at a pH of 7.0 seemed to give a good separation of the PO2. However, as the pH was increased to 7.8 more peaks were separated out demonstrating that a pH of 7.0 gives a clean chromatogram but

is not an ideal pH at which to run the oligo if purity is the goal (see figure 27). Upon completion of a spectral library match, only the oligos separated at a pH of 7.8 or 8.0 give an ideal library match (see table 8). This further points to the fact that a pH of approximately 8.0 is optimal for separation. As a pH of 8.0 is also optimal for DNA stability, this study shows much promise for a good separation of DNA. Although this study needs to be carried further, overall, these results do give conviction to the hypothesis demonstrated by Kato on PO2 oligos that a higher pH gives a better separation of the oligo²³.

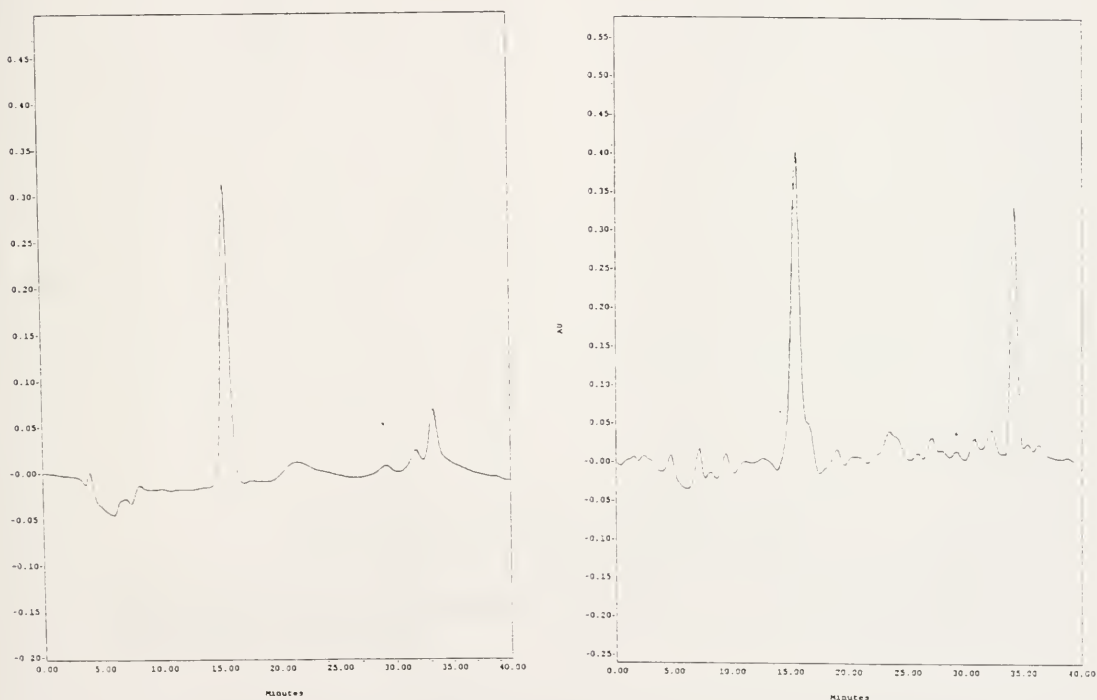


Figure 27: Chromatogram of anti-myc PO2 at (a) pH 7.0 and (b) pH 7.8 using PRP-1 column on Waters 510 pump system Gradient: start at 1mL/min 80%TEAA/20%ACN and switch to 1.5mL/min over 1 min, switch to 70%TEAA/30%ACN over 6 min, switch to 1mL/min over 1 min, hold for 10 min, switch to 60%TEAA/40%ACN over 9 min, hold for 10 min, switch to 80%TEAA/20%ACN over 2 min, hold for 1 min

Performing a spectral analysis on the tritylated peak of each oligo at the extremes of the pH range tested was useful in determining the effect a difference in pH had on the spectra.

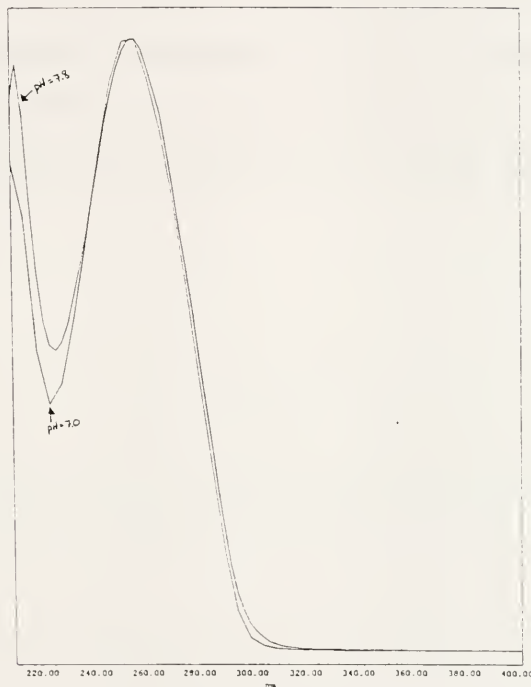


Figure 28: Graph of spectra of tritylated peak of anti-myc PO2 run with 0.1M TEAA at pH 7.0 and 7.8 showing effect of pH on spectra

Figure 28 shows that pH affects not only the chromatogram but also the spectrum. Both of these PO2 oligos were run using the same gradient and concentration, so the only variable in the separation is the difference in pH. By comparing the difference between

the spectra in this overlaid spectra (figure 28) with the difference between the spectra in figure 23, it can be seen that the difference in spectral shape due to a difference in pH is considerable. Therefore, these results contribute to the assertion that a varying pH can have a drastic effect on the library match results²⁷. Thus, the pH needs to be maintained within a close range to minimize these effects.

The buffer comparison between TEAA and TEAB was performed with PO2 and POS modifications at a pH of approximately 8.0. The PO2 chromatograms did not show a great difference in the separation of the peaks although the PO2 run using TEAB buffer does have a slightly narrower PWHH than the PO2 run with TEAA (see figures 19 and 29).

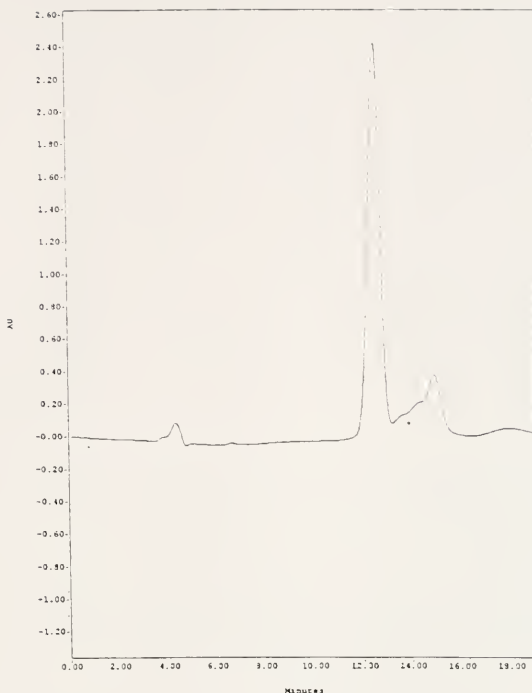


Figure 29: Chromatogram of PO2 oligo run on PRP-1 column of Waters 510 pump system with $T_R=12.460$ min Gradient, pH 7.98: start at 1mL/min 80%TEAB/20%ACN and switch to 1.5mL/min over 1 min, switch to 60%TEAB/40%ACN over 6 min, switch to 1mL/min over 1 min, hold for 8 min, switch to 80%TEAB/20%ACN over 2 min, hold for 1 min

The chromatograms of the POS oligos show some interesting results because the retention times are over 5 minutes apart! (see figures 20 and 30) The PWHH is not ideal for either of the separations; however, the PWHH of the POS using the TEAB buffer was only 3 minutes as compared to a PWHH of 4 minutes for the POS using the TEAA buffer. Therefore, further study of the buffer comparison is warranted to explore these results.

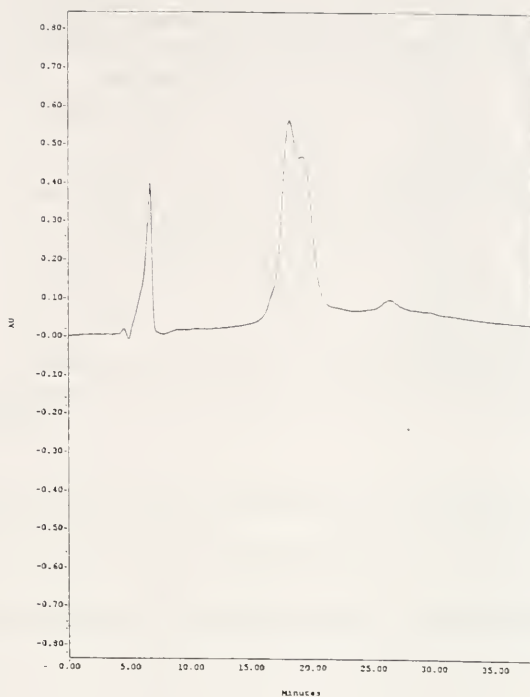


Figure 30: Chromatogram of POS oligo run on PRP-1 column of Waters 510 pump system with $T_R=17.950$ min Gradient, pH 8.22: start at 1mL/min 80%TEAB/20%ACN and switch to 1.5mL/min over 1 min, switch to 70%TEAB/30%ACN over 6 min, switch to 1mL/min over 1 min, hold for 5 min, switch to 60%TEAB/40%ACN over 7 min, hold for 15 min, switch to 80%TEAB/20%ACN over 2 min, hold for 1 min

The spectral library match of the tritylated peak for each oligo was performed to test the resolution and purity of the separation (see table 8).

Library Match of PO2 with TEAA and TEAB		
Anti- <i>myc</i> PO2 at pH 7.8 with TEAA		
Match Angle	Match Threshold	Spectrum Name
0.122	1.638	Tritylated PO2
Anti- <i>myc</i> PO2 at pH 8.0 with TEAB		
Match Angle	Match Threshold	Spectrum Name
1.018	1.716	Tritylated PO2 with TEAB

Table 8: Library match results of buffer comparison on anti-*myc* PO2 at pH of 7.8 and 8.0 with TEAA and TEAB

Both of these matches are ideal since both the match angles are below the match threshold value. However, overall the TEAA buffer seems to give a better match at a pH of 8.0 than the TEAB buffer, since the match angle is much less than the match threshold.

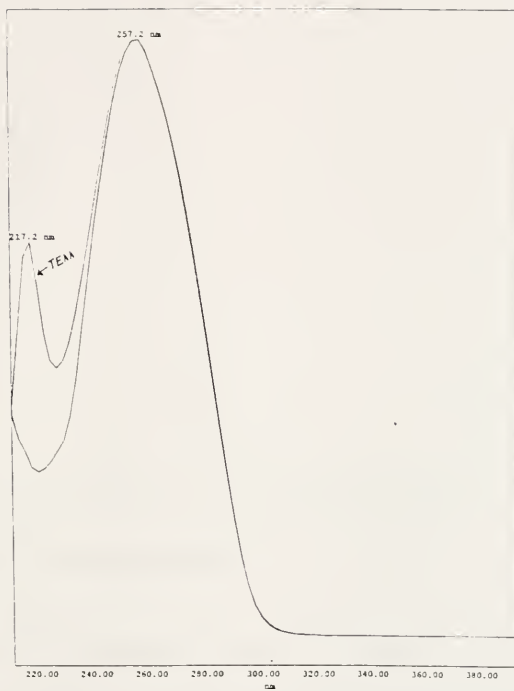


Figure 31: Overlaid spectra of PO2 with TEAA and TEAB

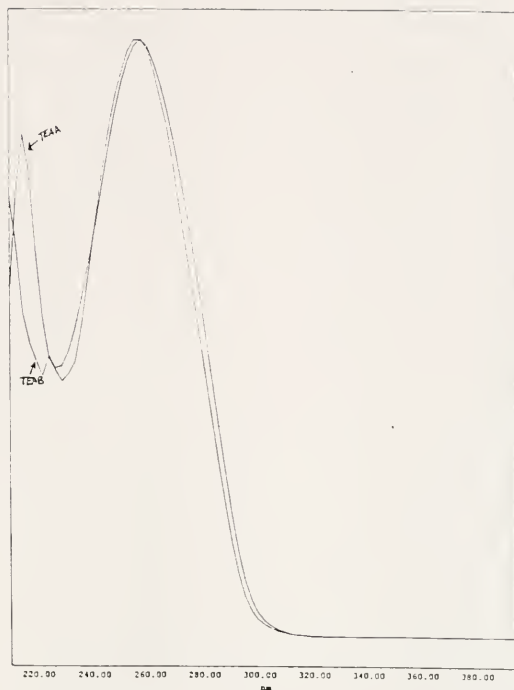


Figure 32: Spectra of POS with TEAA and TEAB

The spectra shown above (figures 31 and 32) show that a change in solvent composition also has an effect on spectral shape, although visual comparison shows the effect on the spectra is not as great as the effect by pH differences.

Although there seemed to be little difference in the use of TEAA or TEAB buffer on the separation, the TEAA buffer is more pH stable and has a longer shelf life than the TEAB buffer as well as being easier to make. This is probably because the TEAB buffer pH is adjusted by dissolving carbon dioxide in it so the pH changes easily as the carbon dioxide diffuses out of the buffer. The pH of the TEAA buffer is adjusted with glacial

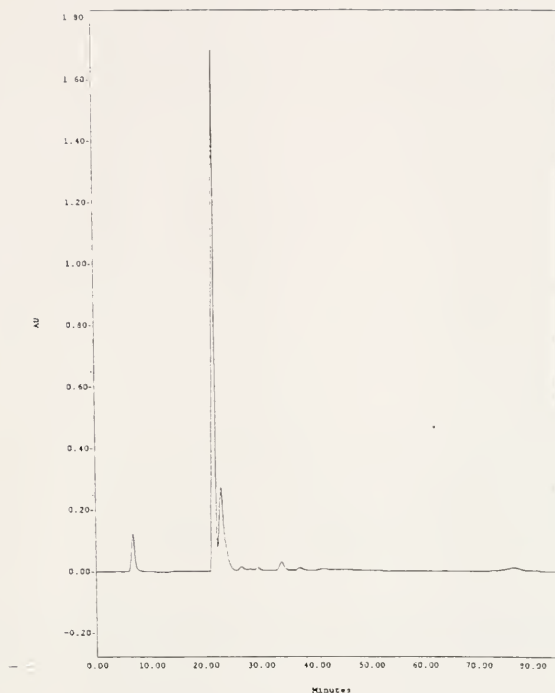


Figure 33: Chromatogram of PS21 using PRP-1 column and Waters 626 pump system at 25°C, Gradient: start at 1mL/min 80%TEAA/20%ACN and hold for 10 min, switch to 70%TEAA/30%ACN over 3 min, hold for 47 min, switch to 60%TEAA/40%ACN over 10 min, hold for 5 min, switch to 80%TEAA/20%ACN over 5 min, hold for 5 min

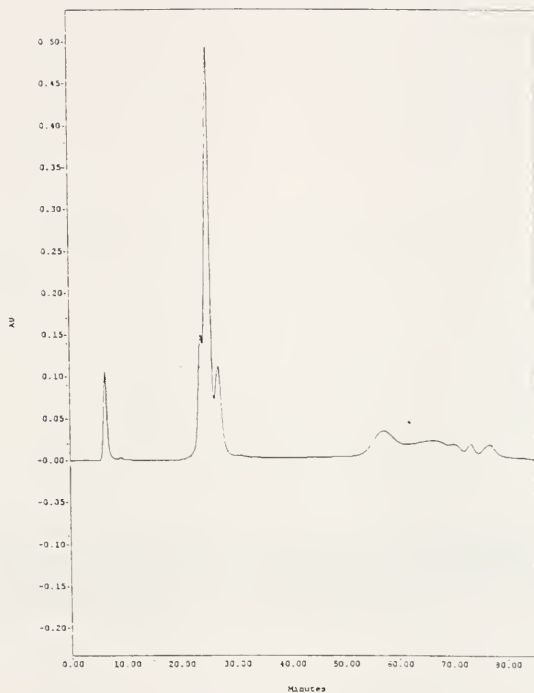


Figure 34: Chromatogram of PS21 using PRP-1 column and Waters 626 pump system at 30°C. Gradient: start at 1mL/min 80%TEAA/20%ACN and hold for 10 min, switch to 70%TEAA/30%ACN over 3 min, hold for 47 min, switch to 60%TEAA/40%ACN over 10 min, hold for 5 min, switch to 80%TEAA/20%ACN over 5 min, hold for 5 min

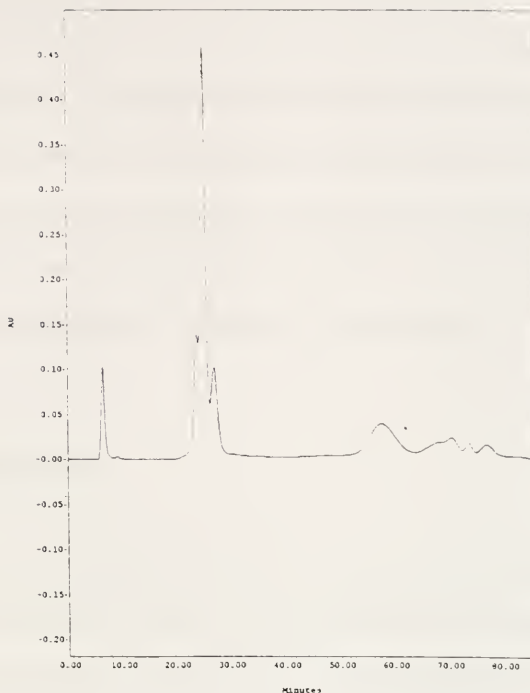


Figure 35: Chromatogram of PS21 using PRP-1 column and Waters 626 pump system at 37°C, Gradient: start at 1mL/min 80%TEAA/20%ACN and hold for 10 min, switch to 70%TEAA/30%ACN over 3 min, hold for 47 min, switch to 60%TEAA/40%ACN over 10 min, hold for 5 min, switch to 80%TEAA/20%ACN over 5 min, hold for 5 min

FURTHER RESEARCH

Spectral Analysis

A new spectral library could be built using chromatograms that have been specifically controlled and run at the same pH, concentration, and solvent compositions.

This will greatly improve the library match results and will aid in the development of a purification method to yield a purer product for cell studies. In addition, if all other separation parameters are kept the same, the effect of instrumentation (different instruments, columns, etc.) on the spectral shape can be determined as well. However, the pH study will still have differences in spectral shape as the pH gets farther away from the pH of the spectra in the library. This may interfere with the library match and make it harder to determine which pH is optimal for each modification.

POS Separation

Recently, during the course of this experiment, some interesting and frustrating results were obtained. The anti-*myc* POS tritylated peak was not appearing as a defined peak as seen previously in these studies. Two different sequences gave drastically different results under the same conditions. The anti-*myc* sequence gave a broad double tritylated peak while the anti-*myc* control sequences resulted in three narrow tritylated peaks eluting from the column isocratically.

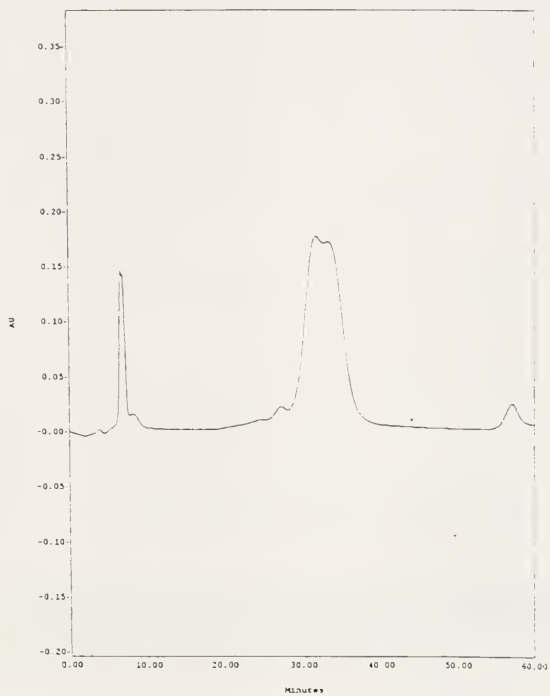


Figure 36: Chromatogram of anti-myc POS using PRP-1 column on Waters 510 pump system
Gradient: start at 1mL/min 80%TEAA/20%ACN and switch to 1.5mL/min over 10 min, switch to 70%TEAA/30%ACN and 1mL/min over 5 min, hold for 30 min. switch to 60%TEAA/40%ACN over 3 min, hold for 7 min, switch to 80%TEAA/20%ACN over 5 min

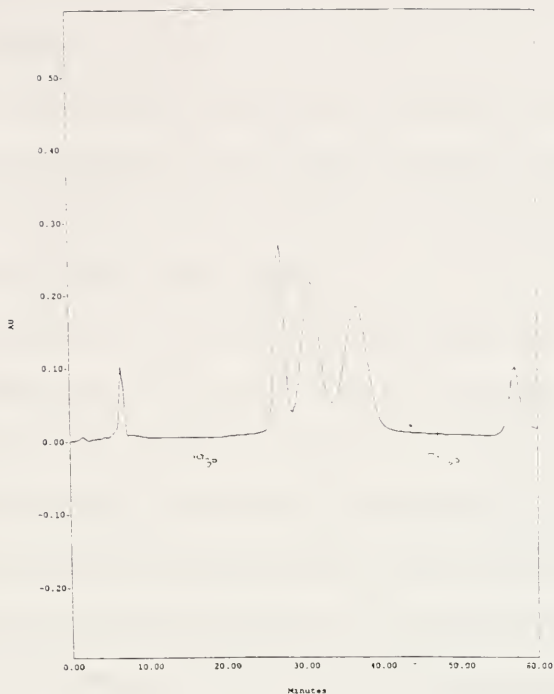


Figure 37: Chromatogram of anti-myc POS control using PRP-1 column on Waters 510 pump system
Gradient: start at 1mL/min 80%TEAA/20%ACN and switch to 1.5mL/min over 10 min, switch to 70%TEAA/30%ACN and 1mL/min over 5 min, hold for 30 min, switch to 60%TEAA/40%ACN over 3 min, hold for 7 min, switch to 80%TEAA/20%ACN over 5 min

This is puzzling because this POS control sequence was synthesized correctly so the only difference between these two sequences is the order of the bases.

To eliminate the possibility of this result being an effect of the older PRP-1 column we ran the POS sequences using the same gradient and buffer conditions. This resulted in the same chromatograms seen before; therefore, the age of the column is not the problem. As an additional test, the anti-myc and anti-myc control PO2 oligos were run on both columns. These oligos gave the same, typical chromatogram on both the

PRP-1 columns used leading to the conclusion that a problem with the HPLC column would not seem valid.

One explanation of these results could be an effect of diastereomers. Since POS oligos are chiral, diastereomers are bound to exist in the mixture. However, if this were the case, 2¹⁴ diastereomers would exist. Since only three peaks have eluted this explanation seems unlikely, but not impossible.

The only difference between the PO2 and the POS analogs is the use of the Beaucage sulfurizing reagent in the synthesis. If this is the source of the problem then this could mean that all of the linkages may not have been sulfurized. This would cause one or more PO2 linkages to be present within the sequence which could affect the hydrophobic character of the sequence just enough to affect its retention time. This seems unlikely because the failure would have to be at a specific linkage for each synthesis to give the same pattern each time. New bottles of Beaucage sulfurizing agent were also used to synthesize the new oligos that gave these same strange chromatograms, which also shows this to be an unlikely hypothesis.

Further research could be done to test the Beaucage reagent by using a new sulfurizing reagent in its place. This new sulfurizing reagent is called 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH) and it has been claimed to be a better sulfurizing agent than Beaucage because EDITH is more soluble and stable in acetonitrile than Beaucage³¹. Therefore EDITH is stable for a longer period of time and will not cause precipitation in the DNA synthesizer.

³¹ Ma, Michael Y X, Jeanne C Dignam, Godwin W Fong, Linhui Li, Steven H. Gray, Biji Jacob-Samuel, & Shaji T George *Nucleic Acids Res.* **25**, 18 (1997) p 3590-93

Variables of Purification

By studying these variables that affect the purification of the oligos, an increase in purity and yield can be accomplished. This achievement in gradient development can then be used to purify oligos quickly and easily for antisense and anti-viral studies in cell culture by Dr. Davies.

Quantitation

Further research will include a study dealing with the quantitation of each modification before and after chromatography. This will aid in determining the percentage of oligo lost in the column. By doing further work on the variables studied in this research and quantitating the yield for each run, a purification method could be determined which would help to increase the yield. The initial concentration of the oligo can be determined using the absorbance determined by a UV/visible spectrophotometer and Beer's Law. The final yield of the tritylated oligo can be calculated in conjunction with the processing method used to integrate the peaks. After integration, the area of the product peak is known and can be used to calculate the percent yield.

pH Study

Further research can be done to extend the pH study by extending the range to a pH of 9.0 as well as exploring the effect of TEAB at different pHs, especially in a further

study of the comparison of POS oligos. By extending the pH range, the range found in the literature will be encompassed and an analysis of the optimum pH can be done. The separation of the tritylated peak from the failure sequences can be directly compared from the chromatograms, using the retention times and the PWHH, and the purity and resolution of the peaks can be determined using the spectral analysis techniques developed in this research. Quantitation of the tritylated oligo can also be done to determine which pH yields the greatest percentage of product.

Instrument Studies

Further research will be done to extend the preliminary instrument study begun in this project. All three PS2 modified oligos, PS21, PS22, and PS23, and the POS and PO2 oligos will be run through the Waters 510 and Waters 626 pump systems to determine if the Waters 626 pump system does not interact with the sulfur modification as is claimed. If all the other parameters of the runs are kept the same and the runs are done in duplicates, then analysis of the chromatogram and spectra using techniques developed in this project, should give conclusive results as to whether or not the Waters 626 pump system is better for the fast separation of thio modified analogs. Quantitation can be used in this study as well to further analyze the biocompatibility of the 626 pump system over the 510 pump system.

Temperature Comparison

Extending the temperature comparison on all of the modifications will also be done as further research to conclusively determine the effect of temperature on the

separation, purity, and yield of the chromatographic run. To do this, a range of temperatures from 25°C to 60°C would be run as values in this range have been cited in the literature. Quantitation and spectral analysis will be performed and the results will then be compared to the results of a similar study on an anion exchange column to see if the separation does increase as the temperature increases²⁴.

Column Comparison

The columns used in the literature for purification of oligos varies, so a comparison between columns would be worthwhile to determine whether a difference in column packing material corresponds to a difference in separation, purity, and yield. To accomplish this all other purification parameters including pH, buffer composition, oligo concentration, and gradient would be kept constant so that only the effect of the column would be seen in the separation, purity, and yield of each oligo. The two columns that would be compared would be the PRP-1 (polystyrene divinylbenzene) column and the C₁₈ column. The PRP-1 column contains a polymeric packing material and the C₁₈ column contains a silica based packing material. Although both columns are said to provide a good separation and yield of DNA and are stable over a wide range of temperatures and pHs, they have not been compared so this analysis will conclude if one column is better than the other.

CONCLUSION

During the past two years, the purification of antisense oligos for this project has evolved from simple gradient development to increase the separation and rate of purification to a multi-step process which adds the analysis of the spectra and purity of each tritylated oligo to the first step. The spectral analysis techniques developed will aid in the identification of many different oligos including sequences with different base compositions and different backbone modifications. Through this research, a foundation has been laid upon which to build by continuing to explore new variables of purification and to move closer to the goal of finding purification methods with higher yields and purity to use in antisense drug therapy.

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